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CIBA FOUNDATION COLLOQUIA ON ENDOCRINOLOGY

VOLUME II

Steroid Metabolism and Estimation

General Editor for the Ciba Foundation

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PREFACE

THE Ciba Foundation is an international centre where workers active in medical and chemical research are encouraged to meet informally to exchange ideas and information. In the two and a half years since its opening in June, 1949, in addition to many part-day discussions, there have been 13 international symposia, each lasting two to four days, attended on invitation by outstanding workers from many countries.

The informality and intimacy of these meetings have permitted discussion of current and incomplete research and stimulated lively speculation and argument. They have also been the occasion for reference to much published and unpublished work throughout the world. The proceedings are now being issued in full, with only the minimum of editing, in order to pass on to a far wider audience the benefits of these meetings. Assembled in book form they present very readably much information not readily available elsewhere.

Nine of the first 13 Symposia form a series of "Colloquia on Endocrinology," dealing mainly with steroid hormone problems. One of these, on Nomenclature of Steroids, has had its conclusions published separately;* of the remaining eight, two are now combined in each of four volumes.

Volume II contains the proceedings of two conferences on closely related subjects, held within a few weeks of each other, and attended for the most part by the same people. The first Book, on Estimation of Steroid Hormones, covers chiefly the determination of urinary metabolites of progesterone, œstrogens, and corticosteroids. The second conference, in Book II, was concerned with the Metabolic Breakdown of Steroids, and attention here was turned mainly to androgens, progesterone and corticosteroids.

*Chemistry and Industry, June 23rd, 1951.

In Volume II the steroid nomenclature used by the Editors has been based on the recommendations of the international group of distinguished steroid chemists at the meeting referred to above. The Editors are greatly indebted to Dr. W. Klyne for help in producing some conformity in nomenclature in this volume, and also for the explanatory note which follows.

STEROID NOMENCLATURE

In any group of naturally occurring compounds, the members first isolated are given trivial names based on their origin (e.g. cholic acid) or their biological activity (e.g. androsterone). Compounds derived from such natural products by degradation, or related compounds, are then given names based on these (e.g. dehydrocholic acid, dehydro*iso*androsterone). Sooner or later, the organic chemists working in such a field find it necessary to introduce a systematic nomenclature. The two names "dehydrocholic acid" and "dehydro*iso*androsterone" illustrate the need for this; the prefix "dehydro" in dehydrocholic acid implies the oxidation of the three hydroxyl groups of cholic acid to keto groups, whilst the same prefix in dehydro*iso*androsterone indicates the presence of an olefinic double bond. The systematic nomenclature usually takes the form of a few basic names for the fundamental hydrocarbons or heterocyclic structures, to which prefixes and suffixes are added in accordance with the general rules of organic nomenclature. Such names, although necessary for systematic chemical work, are often clumsy, and the old trivial names remain in use as well, chiefly for those compounds which have a biological origin or activity.

In a book such as this, it seems desirable to use both the systematic and the trivial names. Some readers will be more interested in the structures of the compounds discussed; others will be more interested in their biological functions. All must, however, have some interest in the connexion between structure and function, otherwise they would hardly be reading the book.

There is, at present, no internationally agreed system of nomenclature for the steroids, but an attempt was made to draft a system at a Conference held at the Ciba Foundation in May-June, 1950. The draft rules have been published in

English (*Chem. and Ind.*, 1951, p. SN1), in German (*Helv. chim. Acta*, 1951, 34, 1680) and in French (*Bull. Soc. chim. Fr.*, 1951, p. i). These proposals were discussed during the meetings of the International Union of Pure and Applied Chemistry in New York in September, 1951; a large measure of international agreement was reached, but adoption of the proposals was deferred until full agreement could be attained. A sub-committee of the I.U.P.A.C., under the Chairmanship of Professor C. W. Shoppee (Swansea) is considering the outstanding points.

In preparing the papers in this Symposium for the Press, the staff of the Ciba Foundation have tried to introduce the "Ciba Foundation Rules" in what it is hoped will be thought a gentle and unobtrusive way. In most cases, the trivial names used by the authors, who are actually working with the compounds, have been retained, with the formal names inserted in parentheses at the first mention in each paper. It is hoped that in future Symposia and in publications elsewhere, authors will gradually forsake the use of many trivial and semi-trivial names, as, e.g., *epi*-androsterone, *cis*-testosterone, and use the systematic names as far as possible. Trivial names will certainly be retained, however, for a few compounds of the greatest biological importance, such as œstrone and progesterone, a list of which is given in the "Ciba Foundation Rules".

3 JAN., 1952 W. KLYNE

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P. M. F. Bishop	*Guy's Hospital, London*
J. R. Brobeck	*Department of Physiology, University of Pennsylvania*
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A. T. Cowie	National Institute for Research in Dairying, University of Reading
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F. Gross	Biological Department, Ciba, Basle
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C. Hamburger	Hormone Department, Statens Seruminstitut, Copenhagen
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R. D. LAWRENCE	King's College Hospital, London
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N B MYANT	University College Hospital, London
W. O NELSON	Department of Anatomy, State University of Iowa, Iowa City
H. NOWAKOWSKI	2nd Med. Universitäts-Klinik, Hamburg-Eppendorf
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C. H SAWYER	Department of Anatomy, University of California, Los Angeles

E. F. SCOWEN	St. Bartholomew's Hospital, London
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E. VAZQUEZ-LOPEZ (deceased)	Imperial Cancer Research Fund, London
F. VERZÁR	Physiological Institute, University of Basle
M. VOGT	Pharmacological Laboratory, University of Edinburgh
P. C. WILLIAMS	Imperial Cancer Research Fund, London
F. G YOUNG	Department of Biochemistry, University of Cambridge
S. ZUCKERMAN	Department of Anatomy, University of Birmingham

List of those participating in or attending the Colloquium on the Effect of Steroids on Local and General Water Distribution, 8th-10th January, 1951.

J. H. Birnie	Morehouse College, Atlanta, Georgia
T. M. Chalmers	Middlesex Hospital, London
D. F. Cole	Department of Anatomy, University of Birmingham
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L. Cournot	Hôpital Broussais, Paris
S. E. Dicker	Department of Pharmacology, University College, London
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Lillian Eichelberger	Department of Surgery, University of Chicago
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Mary Lockett	Department of Physiology, Chelsea Polytechnic, London
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A. S. Parkes	National Institute for Medical Research, London
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MARY PICKFORD	Department of Physiology, University of Edinburgh
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. R WINTON	Pharmacological Laboratory, University College, London
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BOOK I

ANTERIOR PITUITARY SECRETION

FOREWORD

by

A. S. PARKES, Ph.D., D.Sc., F.R.S.

THIS Colloquium dealt with many of the most important problems relating to the anterior pituitary body. The discovery of the dynamic effects of this gland carried with it a logical necessity that co-ordination with the target organs should be reciprocal, especially in the case of the reproductive organs, and further that environmental factors might directly or indirectly influence the anterior pituitary body. Our knowledge of the reciprocal mechanism is very far from complete and many questions still await an answer: how, for instance, is the anterior pituitary body informed of the presence of a fertilized egg in time to change gear for pregnancy? Nevertheless, as the Colloquium showed, a great mass of information has now been accumulated. Some years ago, the anterior pituitary body was referred to as the conductor of the endocrine orchestra That saying has become rather hackneyed, and although true, it is very far from being the whole truth. We now know that the conductor is really just a marionette, activated by different strings pulled, often simultaneously, by various members of the orchestra, as well as by members of the environmental audience. Clearly, this is no ordinary orchestra, and the fact that physiological cacophony is comparatively infrequent shows that the elaborate co-ordination is surprisingly effective. It was the task of the Colloquium to assess how much we know, and even more important, how much we do not know about the mechanisms concerned, and it is to be hoped that the present volume, in which the papers given are presented in full, will be of help to all workers in this field.

PART I

ANATOMY, HISTOLOGY AND CYTOLOGY

CYTOCHEMICAL LOCALIZATION OF THE PROTEIN HORMONES OF THE ANTERIOR HYPOPHYSIS

A. G. EVERSON PEARSE

FROM the anterior pituitary gland with its three classical types of epithelial cell, six protein hormones can be extracted. These are the growth hormone (somatotrophin), lactogenic hormone (mammotrophin), thyrotrophic hormone (TSH), adrenocorticotrophic hormone (ACTH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The problem of which cell produces which of the above hormones is largely unsolved but there is, nevertheless, a general measure of agreement about the source of three of the hormones (growth, lactogenic and FSH), while on the source of the other three there are many opinions and much disagreement. It is usually considered, however, that two of the three cell types, the acidophil and the basophil, between them produce all the known hormones and that the third cell, the chromophobe, is inactive in this respect.

As various functions came to be recorded and ascribed to the pituitary gland, a number of methods were used in an endeavour to establish a relationship between these functions and the individual cell types. Such methods, which are still being employed, fall into six groups:—

(1) Pituitary cytological studies in pathological or physiological states (the classical method).

(2) Pituitary cytological studies in artificially induced states.

(3) Studies of the effects of implantation of whole or specific portions of pituitary glands, or extracts thereof, on the body of the recipient.

(4) Studies of the effects of purified substances extracted from the pituitary gland.

(5) Studies of the effects of substances other than of pituitary origin on the cytology of the gland.

(6) Cytochemical methods.

This paper is primarily concerned with the last of these, but a short survey of the other five, and the conclusions derived from their use, will not be out of place.

Secretory Cytology of the Anterior Hypophysis by the Older Methods

In the classical method of correlating structure with function a comparison is made between the cytology of the hypophysis and the abnormal condition caused by its dysfunction. In this way Benda (1900*a*, *b*) explained the excessive growth in acromegaly and gigantism as due to hyperplasia of the acidophils. After Evans and Long (1921) had produced gigantism in rats by repeated injections of saline extracts of bovine anterior pituitary gland, Benda's reasoning was confirmed when Smith and Smith (1923) found that the hypophysectomized tadpole would show a growth response only when injected with tissue derived from the predominantly acidophil areas of the bovine pituitary. The long series of investigations on Snell's dwarf mice (Snell, 1929; Smith and MacDowell, 1930, 1931; Kemp, 1933, 1936, 1938; Kemp and Marx, 1936, 1937; de Beer and Gruneberg, 1940), in which there is an almost complete absence of acidophils from the anterior hypophysis, make it certain that the origin of the growth hormone is in the acidophils themselves. That this fact is universally accepted is revealed by the relative absence of modern experimental work on the location of the growth hormone.

The relationship between the pituitary gland and the gonads

was not understood until, between 1926 and 1928, the work of Smith in the United States, and of Aschheim and Zondek in Germany, clearly pointed the way to an association between the basophils and the gonadotrophic hormones. Cytological studies on the pituitary glands of castrate animals had been made long before the existence of gonadotrophins became known and most observers were agreed that the number of basophils rose 7–14 days after removal of the gonads and that the cells simultaneously increased in size. On this kind of evidence Cushing (1932) expressed his belief that the sex-maturing principles were elaborated in the basophil elements of the anterior lobe. In the early work on pituitary gonadotrophins it became clear that at least two factors were secreted by the anterior hypophysis and these were named, in respect of their separate properties, FSH and LH. More recently biochemical *evidence, in the preparation of separate pure protein fractions,* called thyla- and metakentrin repectively, has confirmed the existence of these two gonadotrophins. Evidence upon the origin of FSH and LH, derived particularly from work in Groups 1 and 3, is contradictory, although nobody now argues that FSH is produced other than by the basophils. Wolfe and Cleveland (1931) and Wolfe, Phelps and Cleveland (1934) worked on the variations shown by the rabbit's anterior pituitary cells during the sexual cycle and concluded that LH was produced by the acidophils, the basophils alone being responsible for FSH, and Herlant (1943) showed that the rat hypophysis, rich in acidophils after prolonged insulin injections, contained more than the usual amount of luteinizing hormone. This circumstance was taken to favour the production of LH by the acidophils. Severinghaus (1938) in his review of the available evidence agreed that this was best suited by the proposition that the basophils produced FSH and the acidophils LH. Smelser (1944) and Giroud and Martinet (1948*a*, *b*), on the other hand, administered extracts or implants of ox and pig hypophyses to immature female mice or rats, and both sets of authors concluded that FSH and LH were derived from the basophils.

The cause of most, if not all, of the difficulty was revealed by the discovery, by Astwood and Fevold (1939), of a third pituitary gonadotrophin which they named luteotrophin, and by the identification of this with the lactogenic hormone prolactin (Evans, Simpson, Lyons and Turpeinen, 1941). It is now customary to recognize a single hormone producing lactogenic and progestational effects under the alternate names of mammotrophin and luteotrophin. In the case of the former all the evidence, past and present, points to a derivation from the acidophils. In particular the work of Schooley and Riddle (1938) on the pigeon hypophysis strongly supports this view. In this bird a striking degranulation of the acidophils occurs during crop stimulation, a process known to be controlled by prolactin. The experimental work which has the greatest bearing on the derivation of LH and luteotrophin is that of Friedgood and Dawson (1938, 1940, 1942). These authors described a particular type of acidophil in the hypophysis of the cat and rabbit, stained by Heidenhain's azocarmine modification of Mallory's trichrome stain, and this has been the subject of some work of the author reported for the first time in a later part of the paper. As will be seen, this evidence for the derivation of LH from the acidophils is not as good as might appear at first sight.

Consideration of the cytological changes in the anterior pituitary induced by hyperthyroidism, and by thyroidectomy, have not led to agreement as to which cell secretes thyrotrophic hormone, though there is general agreement as to the nature of the cellular changes. In the rat hypophysis, the histological picture following thyroidectomy was described by Severinghaus, Smelser and Clark (1934), by Zeckwer, Davidson, Keller and Livergood (1935), and by Nelson and Hickman (1937). Both types of chromophil cell were found to be affected; the acidophils became degranulated while the basophils increased in size and number. Large vacuolated cells (thyroidectomy cells) appeared, which some workers described as chromophobes but which are correctly regarded as basophils. Marine, Rosen and Spark (1935) confirmed these

changes in the case of the rabbit, but Baker and Everett (1947) denied them in the case of the rat. Severinghaus *et al.* (1934) observed that the administration of thyroxine to rats caused changes in the basophils similar to those induced by thyroidectomy, while the acidophils reacted in the opposite way by becoming hypergranulated. Griesbach (1941) described the changes in the basophils of rats with hyperplastic thyroids induced by goitrogenic diet and concluded, from cytological evidence, that the vacuolated basophils known as goitre cells were active, not degenerate, and might therefore be the source of TSH. Griesbach and Purves (1945) realized that attempts to correlate thyrotrophin production with pituitary cytology failed in severe thyroxin deficiency because the resulting cellular changes affected both acidophils and basophils. They therefore determined the normal thyroxine requirement of thyroidectomized rats and maintained such rats on slightly subnormal levels of thyroxine. Under these conditions the basophils *were increased in number and activity while* the acidophils remained unaltered. They concluded that the basophils were responsible for the secretion of thyrotrophin. A similar opinion was maintained by Zeckwer (1937) who stated that thyroidectomy cells probably produced TSH. Marine, Rosen and Spark (1935) had come to the opposite conclusion from their studies of goitrous and thyroidectomized rabbits, since they believed that depletion of the acidophil granules, coinciding with thyroid activity, suggested the production of TSH by the acidophils. The majority of workers favour its derivation from the basophils.

There is considerable agreement, at the present time, upon the relationship between the pituitary gland and the adrenal cortex; reduction in blood levels of the adrenal cortical hormones is believed to stimulate production of pituitary ACTH with a resulting increase in output and production by the cortex. There is no agreement as yet as to which cell produces ACTH. Earlier evidence, which was derived from studies of the hypophysis in Addison's disease, suggested that the basophils were responsible, but up to the time of Swann's

(1940) review of the pituitary-adrenocortical relationship, experimental work had failed to produce reasonable evidence for or against this hypothesis. Heinbecker and Rolf (1944), working with dogs, produced hypoplasia of the adrenal cortex by hypophysectomy. After cutting the infundibulum and fibres from the paraventricular nucleus, however, the adrenal cortex remained normal while there was a decrease in the number of basophils and a normal number of acidophils in the anterior hypophysis. They concluded that adrenotrophin was produced by the acidophils. D'Angelo, Gordon and Charipper (1948) showed, in the guinea-pig, that starvation caused adrenal hyperplasia, which they presumed to be due to increased adrenotrophin secretion. Under such conditions there was a fall in the number of acidophils with an increase in the number of basophils and chromophobes; they interpreted these findings as indicating an augmentation of circulating ACTH by secretion from the basophils. Pearse and Rinaldini (1950) have shown, in the rat, that starvation increases the number of mucoprotein-containing cells (basophils) by 320 per cent, which corresponds closely to the 260 per cent increase in gonadotrophic potency calculated by bioassay (Rinaldini, 1949). Whether the increased number of basophils which undoubtedly occurs in starvation can be interpreted as giving rise to an increased secretion of ACTH is open to question. Finerty and Briseno-Castrejon (1949) performed unilateral adrenalectomy in rats, which resulted in compensatory hypertrophy of the remaining adrenal and a marked increase in the percentage of acidophils in the hypophyses of treated animals by comparison with controls (50 per cent and 38 per cent respectively). They believed, on this account, that the acidophils produced ACTH.

From all the above work, the majority of which falls into Groups 1, 2 and 3 of the classification of methods given above, it is now usually concluded that growth hormone and the lactogenic hormone (prolactin, luteotrophin) are derived from the acidophils while FSH comes from the basophils. The origin of the other three hormones remains uncertain. In

spite of the relatively strong evidence to [illegible] from [illegible] [illegible] confidently attributed to the latter. Fig. 1, below, is taken from an illustration by *Fuller Albright* (1948) which sets forth his views on the derivation of the various hormones. These views were [illegible] clinical [illegible] said

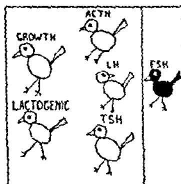


FIG. 1. The secretory cytology of the anterior hypophysis as determined by non-cytochemical methods (Acidophils white, basophils black.) *After Albright* (1938).

Albright, but LH is looking over his shoulder because he is not quite sure of his place. Such is the position when we come to apply the methods of cytochemistry to the investigation of the problem.

The Cytochemistry of the Anterior Hypophysis

Herlant (1942, 1943) was the first to use the cytochemical approach. He employed a metachromatic staining technique and claimed that he could specifically demonstrate the gonadotrophic hormones in the [illegible] on the [illegible] LH) be[illegible] [illegible]oproteins and that such

substances are capable of inducing metachromasia with dyes like toluidine blue. Subsequent work by Catchpole (1947, 1949) and by Pearse (1948, 1949) has made further use of the mucoprotein nature of the gonadotrophins, coupled with newer techniques of investigation. The protein hormones of the anterior pituitary gland can be divided into two groups, first on the basis of their carbohydrate content and secondly on their isoelectric points (I.E.P.). When this is done, as in Table I opposite, it can be seen that FSH, LH and TSH fall into Group 1 while growth hormone and prolactin (luteotrophin) fall into Group 2. The pituitary gland thus produces two main classes of protein: one acid, basophil and carbohydrate-containing (mucoprotein); the other basic, acidophil and carbohydrate free (simple protein). The prime examples of the two classes are FSH and growth hormone. There are two anomalies which mar the perfection of this scheme. First, the high I.E.P. reported for swine LH by Shedlovsky, Rothen, Greep, van Dyke and Chow (1940), and secondly, the fact that ACTH does not conform to the major division between the two groups. The preparation referred to by Shedlovsky *et al.* was a pure protein, with an isoelectric point at pH 7·85, which "stimulated the interstitial tissue of the testis or ovary and caused the formation of corpora lutea provided that maturing follicles were present." It was free from follicle-stimulating activity but Greep, van Dyke and Chow (1942) observed in respect of it that "a preparation of pure sheep ICSH (LH) proved consistently more effective in ovulating does (rabbits) than did hog metakentrin (LH)." It seems likely that the fraction isolated by Shedlovsky *et al.* consisted of luteotrophin and that in this sense only could it be regarded as a luteinizing hormone.

Because of the anomalous position of ACTH in Table I, the histochemical methods employed by Pearse (1949, 1952) could not be expected to achieve its localization in the anterior hypophysis. It could be present in the beta granules without causing an appreciable alteration in their chemical and physical properties. Similarly, if present in the alpha

granules, no alteration would be expected unless the amount was greater than that of the simple protein, with high I.E.P., of which these granules consist. In the case of the other hormones, however, cytochemical localization is theoretically possible. All reported analyses of protein fractions from the hypophysis corresponding to FSH, LH and TSH record the presence of hexose and hexosamine in significant quantities. These substances can be demonstrated cytochemically without difficulty. The periodic acid-Schiff (PAS) reaction of McManus (1946) and Hotchkiss (1948) was used by both Catchpole and by Pearse to demonstrate the presence of hexose and hexosamine-containing mucoproteins in the

Table I

Carbohydrate	*Low I E P.*	*High I E P*
Positive	FSH, LH, TSH } Group I	—
Negative	ACTH	Growth, Lactogenic } Group II

anterior hypophysis. Pearse (1949) showed that such material was present exclusively in the basophils and in some cells, classified as chromophobes by older staining techniques, which he regarded as belonging to the basophil series. This was true not only of the human gland but of the other species studied (rat, guinea-pig, rabbit, mouse, sheep, goat). The PAS reaction has been considered to be specific for the 1:2-glycol linkage which occurs in a variety of tissue components, but whatever the mechanism of the reaction it can be shown to be capable of revealing substances in five main groups. These are: (1) polysaccharides (glycogen); (2) mucopolysaccharides (mucins, hyaluronic acid, heparin); (3) muco- and glycoproteins (mucoids, gonadotrophic and thyrotrophic hormones, fractions of serum albumin and globulin); (4) glycolipids (kerasin, phrenosin); and (5) phospholipids (lecithin, cephalin).

When the method, with suitable counterstains, is applied to the pituitary gland the basophils are deeply red, due to the colour of the individual beta granules and the true chromophobes are at the most pale pink. By means of various complementary histochemical tests it has been shown by Pearse (1949, 1952*a*) that this colouration of the beta granules is due to their content of mucoprotein (though neutral mucopolysaccharide cannot be specifically excluded) and not to substances in any of the other groups mentioned above. Catchpole (1949), moreover, has shown in the case of the rat hypophysis that the presumed mucoprotein which stains with PAS has the same point of minimum solubility in buffers as the mucoprotein FSH.

The alpha granules, in all species so far examined, have been found free from any traces of redness when the PAS reaction is applied and they stain yellow with the orange G counterstain used in the Trichrome-PAS method (Pearse, 1950). This means that they contain certainly no more than a trace and probably no reacting polysaccharide (1:2-glycol) whatsoever. It can be shown that the PAS reaction will easily appreciate 1 per cent of polysaccharide. From figures given by Seibert, Pfaff and Seibert (1948) for the albumin and globulin fractions of normal human serum, to which figures for fibrinogen have been added, it has been calculated that normal human plasma contains about 1·4 per cent of polysaccharide (dry weight). The colour of lipoid-free plasma, stained by the PAS method, is a recognizable pink.

As a result of these cytochemical investigations it can be stated that the mucoprotein hormones of the anterior hypophysis (FSH, TSH, LH), provided that they are not synthesized extracellularly from an intracellular carbohydrate-free precursor, can only be derived from the mucoprotein beta granules of the basophils. The growth and lactogenic (luteotrophic) hormones, on the other hand, are known to be composed of basic, or relatively basic, simple proteins and such proteins have been shown to be present in the alpha granules of the acidophils. Using Albright's mode of expression,

Fig. 2 shows the secretory cytology of the hypophysis as determined by cytochemical means. The left hand column refers to the acidophils, the right hand one to the basophils. In the middle, because no proof one way or the other can be offered, stands ACTH.

Comparison Between Cytochemical and Other Evidence

It may reasonably be said that the non-cytochemical evidence against the derivation of FSH from the basophils is

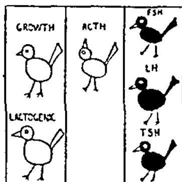


FIG 2. The secretory cytology of the anterior hypophysis by cytochemical methods. For comparison with Fig 1.

negligible and on this score the cytochemical methods in Group 6 are in complete agreement with the other groups. In the case of TSH, the non-cytochemical evidence against its derivation from the basophils is slender and I consider that the cytochemical findings, in conjunction with the observations of Griesbach and Purves (1945), indicate conclusively that this hormone is produced by the basophils. Only in the matter of the luteinizing hormone (LH) does the cytochemical evidence conflict sharply with the non-cytochemical evidence offered by Friedgood and Dawson. Much of this difference is due to the fact that not one, but two hormones capable of exerting luteinizing effects are secreted by the anterior hypophysis.

Though these are chemically separate and distinct hormones there is some difficulty in distinguishing their several effects biologically. The course of events in the hypophysis of the rabbit following coitus has been built up from a number of observations. Hill (1934) found that the anterior pituitary gland of the normal female rabbit contained 1,560 rabbit-ovulating units of gonadotrophin, while half an hour after coitus this had fallen to 1,220 units (20 per cent decrease) and after 24 hours to 210 units (87 per cent decrease). It is known that in the rabbit ovulation is not induced by FSH alone but by FSH and LH. Fee and Parkes (1929) showed that while hypophysectomy of the female rabbit (by decerebration) one hour after coitus would prevent ovulation, the same operation performed later than this had no such effect. Sawyer, Markee and Hollinshead (1947) were able to inhibit ovulation in the rabbit by interruption of the humoral pathway from the hypothalamus with the adrenergic blocking reagent dibenamine. This was only possible if the dibenamine was injected less than 3 minutes after coitus. We know, therefore, that a minute or so after coitus the stimulus for discharge of gonadotrophin reaches the anterior pituitary, that within half an hour 20 per cent of the total gonadotrophin has been discharged and that in about one hour the amount of LH secreted is sufficient to produce ovulation. We do not know how long this discharge of LH continues nor yet (conclusively) from which type of cell it is produced. Friedgood and Dawson scarcely mentioned the basophils in their descriptions of the hypophyseal changes following coitus, so that it seemed essential to repeat their work and particularly to examine the part played by these cells.

Cytology of the Rabbit Hypophysis after Coitus

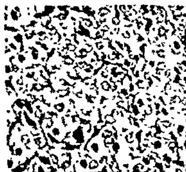
The essential observation made by Friedgood and Dawson on the rabbit and cat hypophysis was that within 30 minutes of coitus there appeared in the gland large numbers of a modified acidophil (Fig. 5), whose granules have a particularly great affinity for the dye azocarmine. This dye can be

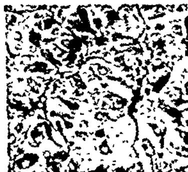
removed with ease from the granules of the normal acidophils (Fig. 3). The name carmine cell, later (Dawson, 1946) carminophile, was applied to them. Though they were normally present only in small numbers, a particularly striking rise in carmine cells occurred in two conditions, (1) immediately following coitus and (2) during late pregnancy and early lactation. Friedgood and Dawson initially traced the origin of LH to the carmine cell, and therefore to the acidophil, by correlating the hypophyseal changes following coitus with the levels of serum gonadotrophin The carmine cell reached its zenith between 1 and 3½ hours post-coitum while degranulation was observed to take place 4 hours after coitus and to be complete after 14 hours. This latter period was observed to coincide with the maximum levels of gonadotrophin in the serum. They concluded (1942) that the carmine cell contained a luteinizing hormone essential for the initial maturation of the follicle and that its subsequent degranulation represented the secretory phase. A further conclusion from this work, and that of Dawson (1946) on the effect of lactation on the carminophiles, was made by Friedgood (1946). He stated that circumstantial evidence indicated that prolactin was derived from the carmine cells.

My own findings agree with the particularly accurate observations of the original authors in the matter of the carmine cell. The poorly staining normal acidophils, shown in Fig. 3, became converted in 1½ hours almost entirely into fully granulated carmine cells, of the type seen in Fig. 5. When the basophils were examined, however, a change only chromatically less striking than that affecting the acidophils was observed. It is necessary at this point to refer briefly to the work of Severinghaus (1935, 1938). This author described in the acidophils and basophils of the human hypophysis a cycle of degranulation and restoration accompanied by nuclear changes of a distinct and easily recognized type. Using his own modification of Mallory's trichrome stain, Severinghaus found that the nuclei of degranulating acidophils assumed an overall blue colouration towards the end of the

secretory part of the cycle while at a similar stage in the basophils the nuclei became bright red. He described these nuclear changes as pyknotic, a use of the word with which I do not agree. The essential change (Pearse, 1952*b*) consists of a diminution in the stainable quantity of acid and therefore basophil protein in the chromatin threads, together with an unmasking of, or an increase in, the acidophil proteins of the ground substance. Why in the case of the acidophils the increased acidophilia of the nucleus should express itself as an affinity for aniline blue, while in the basophils it shows as an affinity for the red acid dyes (acid fuchsin, azocarmine) has not been explained.

When the basophils from a doe half an hour after coitus are compared with those of a control female rabbit in œstrus (Fig. 4), the characteristic alteration of the nuclei described by Severinghaus is seen to have increased from less than 1 per cent to about 30 per cent. One hour after coitus over 60 per cent of the nuclei of the basophils show this change (Fig. 6). Instead of being composed of fine purple-staining chromatin threads, often enclosing a single red nucleolus (Fig. 4), the whole nucleus has acquired a strong affinity for azocarmine so that it stains bright red. After 3 hours the percentage of cells showing the nuclear change is still high but is diminishing both numerically and qualitatively, with evidence of a return to the normal chromatin pattern in many of the cells. At this stage degranulation of the basophils, which has been proceeding in the two previous stages, becomes quite obvious (Fig. 8). After 6 hours (Fig. 7), the majority of basophils are refilling with beta granules though some are still degranulated and others have nuclei intermediate between the degranulating type and the normal. The carmine cells, on the other hand, can be observed to be degranulating at 3 hours post-coitum as judged both by diminution in size and granule content and by the presence of the blue nucleus of degranulation described by Severinghaus. At 6 hours post-coitum they are extensively degranulated and the nuclear change is still visible (Fig. 7). Table II shows the results given by Friedgood and Dawson





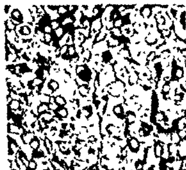


Fig. 3 (*top left*). Normal acidophils in the hypophysis of a rabbit in œstrus. No carmine cells are visible. Heidenhain's Azocarmine method × 300.

Fig. 4 (*top right*). Normal basophils in the hypophysis of a rabbit in œstrus. Granulation is not particularly dense. Note nuclei with single (red-staining) nucleoli. Heidenhain's Azocarmine method × 300.

Fig. 5 (*bottom left*). Darkly staining carmine cells in the hypophysis of a female rabbit 1½ hours after coitus. Heidenhain's Azocarmine method × 300.

[Editor's Note. Figs. 3-8 were originally shown in colour and it is regretted that it has not been found possible to print them here in colour.]

[*To face page* 14

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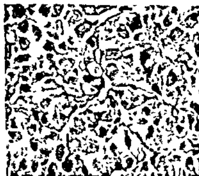
(1938) for the carmine cell change in the rabbit hypophysis together with my own results, from a small series of 18 rabbits (3 controls and 3 at each time interval), expressing the degranulation of the basophils in a similar manner. Both the nuclear change (acidophilia) and frank loss of granules have been taken as indicators of degranulation.

Table II

Time post-coitum (hours)	*Carmine cell reaction*	*Basophil degranulation*	*Remarks*
½	+ ++++ ++++	++ ++++	Basophil degranulation already under way. 20 per cent drop in pituitary gonadotrophin
¾	++ +++	No material	—
1	++ +++	+++ ++++ +++++	Basophil degranulation increasing. Ovulation proceeds post-hypophysectomy.
1½	++++ +++++ ++++++	++++ +++++ ++++++	Maximum degranulation of basophils.
2½	++ ++++	No material	—
3–3½	++ ++++	++ +++ ++++	Degranulation of carmine cell begins
4½	+ +++	No material	—
6–8	++	+ +++	Basophils regranulating. Carmine cells still degranulating

Columns 1 and 2 are taken from Friedgood and Dawson (1938).
Column 3 represents author's observations

I conclude from these observations (1) that the secretion of sufficient gonadotrophin (known to be mainly LH) to produce ovulation coincides with the beginning of the period of maximal degranulation of the basophils and (2) that at this



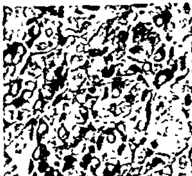


Fig 6 (*top left*). Basophils in the female rabbit hypophysis one hour after coitus. The Severinghaus change affects nearly two-thirds of the nuclei Heidenhain's Azocarmine method 300

Fig 7 (*top right*) The female rabbit hypophysis six hours after coitus Left, regranulating basophils, some showing the "intermediate" state of the nuclei. Right, degranulating carmine cells, some showing the (blue) nucleus of degranulation Heidenhain's Azocarmine method. × 300.

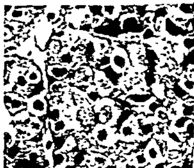


Fig 8 (*bottom right*). Degranulated basophils in the female rabbit hypophysis three hours after coitus Severinghaus change still visible in the majority of nuclei Heidenhain's Azocarmine method.

(4) Cytochemical methods support the source of the simple protein growth and lactogenic hormones as the alpha granules of the acidophils, but give no indication, at present, of the source of ACTH.

(5) The outstanding non-cytochemical evidence for the derivation of LH from the acidophils is the work of Friedgood and Dawson on the carmine cell. This work has been repeated in the rabbit, and it has shown that sufficient LH to induce ovulation is secreted during the period of maximum basophil degranulation. No degranulation of the carmine cells can be observed at this time.

(6) The appearances in the post-coital rabbit hypophysis support rather than deny the derivation of LH from the basophils.

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time, and indeed until about 3 hours post-coitum, the carmine cells show no evidence of degranulation whatsoever. In view of these conclusions it is likely that not only the mucoprotein FSH but also the mucoprotein LH (metakentrin) are secreted by the basophils and the discrepancy between cytochemical and cytological reasoning is removed. This repetition of the work of Friedgood and Dawson does not prove that LH is derived from the basophils, merely that it could be and very likely is. In conjunction with the cytochemical evidence given above, the possibility becomes a certainty. As regards the true function of the carmine cells, the findings of cytochemistry agree very well with the hypothesis that they are responsible for the secretion of the lactogenic hormone (luteotrophin).

Summary

(1) The methods which have been used in attempts to correlate the secretory functions of the anterior pituitary gland with its cytology fall into six groups. The first five of these may be aggregated under the single heading "non-cytochemical." The sixth group comprises the cytochemical methods with which this paper is chiefly concerned.

(2) The problem of which of the cell types of the anterior pituitary gland produces which of the six known protein hormones is reviewed in the light of the non-cytochemical evidence. This broadly suggests that growth and lactogenic hormones are produced by the acidophils and FSH by the basophils. In respect of the other three (LH, TSH, ACTH) the evidence favours production of the first by the acidophils, the second by the basophils and the third by either.

(3) Cytochemical methods, capable of revealing smaller amounts of carbohydrate (hexose and hexosamine) than those which are found in any of the three mucoprotein hormones (FSH, TSH, LH), indicate that none of them is present in the acidophils (alpha granules) unless in the unlikely form of carbohydrate-free precursors. They can only be present in the beta granules of the basophils.

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DISCUSSION

Parkes: Were you able to get your human material in reasonably fresh condition?

[illegible] e of glands less than [illegible] 1½ hours. With the [illegible] nges in the majority [illegible] I usually throw out *any over that period*

Parkes. What do you imagine causes the continued secretion by the rabbit pituitary after an amount adequate to cause ovulation has been poured out?

Pearse: I suppose that goes on for at least an hour and a half after it's necessary.

Parkes: The pituitary very soon has to start thinking about the building up of corpus luteum. Have you managed to trace any changes corresponding with this phase?

Pearse: I think that the work of Friedgood and Dawson suggests that luteotrophin is secreted from the third hour onwards in large quantities, and FSH may still be excreted at that time. It's very difficult to say from this kind of preparation, but I take it that the output of luteotrophin ceases very shortly after three hours, and that the excess that's put out is rather typical of the body—it never seems to do anything without overdoing it.

Tuchmann-Duplessis: I would like to ask Dr. Pearse what he thinks about the McManus reaction? Do you think that this is a specific reaction for the mucoprotein of the pituitary?

Pearse. I think it's probably the most unspecific reaction in the world. It never fails to stain anything. But it's a very useful indicator of these materials. It never fails to stain a material that contains more than about 1 per cent of hexose and hexosamine.

[illegible] cells. Basophil, as far as I am concerned, is [illegible]

[illegible] basophils. There are in fact two kinds of mucoprotein granules seen in the pituitary, the true classical beta-granule and this other granule which I have sometimes called the gamma-granule—I don't much like

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THE PHYSIOLOGICAL MEANING OF THE HISTOLOGICAL PICTURE OF THE ANTERIOR HYPOPHYSIS CHARACTERISTIC OF CASTRATION, PREGNANCY AND THYROIDECTOMY

L. DESCLIN

The ultimate purpose of every cytologist studying the anterior lobe of the pituitary is to discover in what cells the various hormones elaborated in this organ are formed and to characterize the aspects of these cells related to the rate of elaboration and release of these secretory products. The difficulty of this task has been stressed by many authors (Severinghaus, 1936, 1937; Romeis, 1940) and results mainly from the multiplicity of the pituitary hormones contrasting with the relative uniformity of the cellular pattern in the anterior lobe.

Three different cellular types only are generally present in the anterior lobe of vertebrates. Subcategories of these cell types seem to be due to species differences without character of generality or do not aid much at present for the understanding of the physiological processes. It is clear that pure cytological research cannot give the solution of such problems, which may only be solved through the comparison of physiological data with the details of morphological observations. Our present knowledge of the functions of the different cells of the anterior lobe rests mainly on this comparison.

The study of well defined structural changes, such as those resulting from castration, pregnancy or thyroidectomy, have contributed the most important part of our knowledge of these problems.

It is my purpose to report here the results of experiments performed recently in my laboratory concerning the

these designations—but the difference between them chiefly seems to be a matter of polymerization. The beta-granule, because it appears to [illegible]

but I can't help it.

Sawyer: Friedman and others have shown that the pituitary is maximally depleted of LH at about 24 hours after coitus. Do you get a second depletion in the basophil granules following the initial drop?

Pearse: I didn't examine them after 8 hours.

Nelson. One might interpret your findings on the basis that the delta cells were successively releasing two different gonadotrophins, and that both are therefore participating in the phenomenon of ovulation.

Pearse. Yes. Successively, I think.

horn was implanted regularly in one of the ears. The animals were killed two months later.

It is now a well established fact that the œstrogen secreted by an ovary transplanted to the spleen is inactivated in the liver. This has also been the case in our experiments; if the ovary had been implanted at this site, the uterine horn implanted in the ear of the castrated male under these conditions remains completely atrophic. Its aspect was entirely different when the ovarian graft was located in the ear. In this case the piece of uterus was much bigger, the epithelium was definitely higher and many glands could be seen in the mucosa. The œstrogenic stimulation was obvious.

This situation was also reflected in the study of the hypophysis of these two groups of animals. Whereas the animals bearing an ovarian graft in the spleen had a pituitary of the castration type, with very numerous castration cells, the anterior lobe of those grafted in the ear did not show these hypertrophied basophils. They were in fact not completely normal and, from time to time, a hypertrophic basophil could be found.

The comparative cell counts in castrated controls, and in the two series of experimental animals are given in Table I, which shows that whereas the structure of the anterior lobe has not been influenced by the ovary implanted in the spleen, it has been influenced by the ovary implanted in the ear. In this case the number of basophils is much reduced. Nevertheless the correction is not complete. The figure of 14 per cent found in these animals is much higher than in our normal males, where we found $10 \cdot 7 \pm 0 \cdot 22$ per cent. They could also not be considered as feminized. Our female controls showed a figure of $5 \cdot 09 + 0 \cdot 11$ basophils. Contrasting with this, the basophil rates remained very high in the series where the graft was located in the spleen, as high as in the non-grafted castrated male. On the other hand, the percentage of eosinophils in the different series remained constant and their degranulation was least in the case of the intrasplenic graft.

significance of these structural changes, and they do not entirely conform to current views on this matter.

The study of the pituitary of castrated animals has contributed much to the idea that the gonadotrophic hormones of the pituitary are elaborated in the basophils of the anterior lobe. This is at present one of the best established facts regarding correlation of cellular activity with physiological function. It rests on a great deal of evidence: increase in number and size of the basophils after gonadectomy, and their transformation into particular cells, the so-called castration cells, having a vacuolated cytoplasm with a hypertrophied Golgi apparatus (Fichera, 1905; Addison, 1917; Severinghaus, 1932; Desclin, 1933, 1934, etc.); parallel increase in the gonadotrophic content of the pituitary after castration, as shown through the implantation of the anterior lobe in immature receptors (Engle, 1929; Evans and Simpson, 1929; Severinghaus, 1932; Nelson, 1935; Lauson, Golden and Severinghaus, 1939); excessive release of gonadotrophic hormones after castration, as shown by the experiments of parabiosis (Martins and Rocha; Hill, Witschi *et al.*). These last experiments and other researches on ovarian grafts in the castrated male (Lipschütz *et al.*, 1925; Smelser, 1933; Pfeiffer, 1934; Bartschi and Ponse, 1934, Goodman, 1934; Pfeiffer, 1936; Deanesly, 1938) have led to the consideration that the basophils are the site of formation of the follicle stimulating hormone only, whereas the luteinizing hormone should be elaborated within the eosinophils (Severinghaus, Wolfe, Herlant).

In the last few years, Dr. Kempf in my laboratory has made a comparative study of ovarian grafts in the ear and in the spleen of male castrated rats At the same time, he has made a very complete study by the method of cell counts of the pituitaries of these animals. The rats used were either immature or adult rats. The immature ones were 30 days old when the graft and castration were performed. The adults were 4 months old at the time of operation. To detect the œstrogenic activity of the ovarian graft, a piece of uterine

The comparative study of the structure of the implanted ovaries after two months was very interesting. For the grafts in the ear, our results are in complete agreement with those of previous studies by others in the male (Moore, 1919, Goodman, 1934; Pfeiffer, 1936; Deanesly, 1938). They show only follicles or follicle cysts, but no corpora lutea. When the ovary has been implanted into the spleen, the picture after two months is entirely different. As a rule, they are much bigger and completely filled up with a tremendous number of corpora lutea. One can find sometimes 30 of them on a single preparation.

This difference in the structure of the splenic graft as compared with that of the implants in the ear must obviously be related to the difference in the structure of the pituitaries in these two conditions.

To make sure that a particular influence of the spleen itself could not be the cause of these differences, we recently compared three series of animals implanted with two ovaries. Male rats were 7 months old at the beginning of the experiment. In one series both ovaries were grafted in the ears and castrated the same day. In another group similarly castrated, both ovaries were grafted in the spleen, whereas in a third series one ovary was implanted in the ear, the other in the spleen. In the two first groups, the results were exactly the same as those of Dr. Kempf, showing only follicles and no corpora lutea when the graft had been made into the ear; showing on the contrary very numerous corpora lutea in both ovaries grafted in the spleen. The pituitary showed equally a pure castration picture in this latter case; whereas in the first the castration picture had almost completely regressed. But in the third group of animals, where one ovary was implanted in the ear, the other one in the spleen, both grafts showed the same structure and only contained follicles. The graft in the ear had exerted its influence on the anterior lobe, which was corrected to a considerable extent. We may therefore conclude that the picture of the ovary in the spleen is the exact expression of the activity of the anterior lobe. But we

Table I

PERCENTAGE OF THE DIFFERENT CELL TYPES IN THE ANTERIOR LOBE OF CASTRATED RATS

In some of them an ovary has been implanted either in the spleen or in the ear Percentages for normal females are also given

Site of implantation	*Chromophobes*	*Eosinophils*			*Basophils*		
		Granulated	*Degranulated*	*Total*	*Granulated*	*Degranulated*	*Total*
Castrated male controls .	47 06 ±0 78	31 80 ±0 34	1 30 ±0 13	33 10 ±0 86	5 66 ±0 14	14 18 ±0 78	19 84 ±0 91
Castrated males grafted in the spleen	46 60 ±0 40	31 30 ±0 20	0 82 ±0 42	32 13 ±0 24	6 23 ±0 14	15 01 ±0 52	21 25 ±0 60
Castrated male grafted in the ear . .	51 67 ±0 54	32 18 ±0 24	1 81 ±0 38	33 99 ±0 51	4 96 ±0 35	9 38 ±0 36	14 34 ±0 48
Normal females . . .	60 64 ±0 13	29 85 ±0 12	4 40 ±0 06	34 26 ±0 13	1 19 ±0 06	3 90 ±0 1	5 09 ±0 11

that this LH comes from the eosinophils? The evidence is not convincing. In pseudopregnant rats and in rats treated with œstrogen, the eosinophils are not the only elements showing evidence of activity. The basophils are intensively degranulated and show signs of active secretion, also enlargment of the Golgi area.

Similar basophils are present in the pituitary of the lactating rat. In this case we have shown that this active state of secretion persists after castration, providing that young are suckling (Desclin, 1936). But we found also that in an ovary grafted in the kidney under these conditions, no follicular development can be found. These very active basophils therefore do not release FSH but could very well be the site of LH secretion in accordance with the facts reported in our previous experiments. As for the significance of the degranulation and activity of the eosinophils during pregnancy, pseudopregnancy and lactation, much of the evidence resulting from our personal observations during lactation (Desclin, 1936, 1945, 1947) and from other authors in mammals (Everett and Baker, Dawson) and in birds (Schooley and Riddle) indicate that they are elaborating and releasing prolactin.

Besides their rôle in the elaboration of the gonadotrophic hormones, the basophils are also thought to be the site of formation of the thyrotrophic secretion of the anterior lobe.

The most impressive evidence here comes from the so-called thyroidectomy picture. It has been known for a long time that the removal of the thyroid in the rat determines profound structural changes in the pituitary. These alterations appear very rapidly and consist in an intense degranulation of the eosinophils accompanied by an increase in number and volume of the basophils. These last cells show very quickly numerous vacuoles and are similar to the so-called castration cells found a relatively long time after the removal of the gonads. Much controversy has arisen about their exact nature. Some authors have considered them as identical with the castration cells (Severinghaus, etc.), others have stressed the differences

must also come to the conclusion that the pituitary after castration, if it is completely unchecked and liberated from the control of the ovary, releases notable quantities of luteinizing hormone.

We should like to stress again the fact that the only difference in the pituitary picture between the animals grafted in the spleen and those implanted into the ear affects the basophils. The eosinophils remain unchanged in both conditions. We must therefore conclude that the basophils are the site of elaboration of both gonadotrophic hormones FSH and LH.

This conclusion is in opposition to the current view, advocated particularly in the last few years by Severinghaus, Wolfe and recently by Herlant. Most of the evidence in favour of this theory came from the study of the pituitary in pseudopregnant animals and in female rats treated with œstrogen. Their pituitaries have been found to show a degranulation of the eosinophils, which I have myself observed (Desclin and Brouha, 1931; Desclin, 1933, 1934, 1935). These eosinophils show evident signs of great activity: increased number of mitochondria (Severinghaus), enlargement of the Golgi area (Severinghaus, Desclin) and basophilic ribonucleic inclusions (Desclin, 1935, 1936, 1940; Dempsey and Wislocki, Wolfe). They are found also in the so-called pregnancy pituitary. Their presence is always coincident with the presence of active corpora lutea in the ovary, and has been interpreted as a manifestation of the release of LH.

In particular, the experiments of Hohlweg (1935), by which he could provoke the precocious apparition of corpora lutea in the ovaries of immature rats under the influence of œstradiol injections and produced a similar degranulation of the eosinophils in the anterior lobe, contributed much to the success of this theory. It might be interesting to say on this occasion that Dr. Kempf could also obtain the luteinization of ovarian grafts in the ear of the male rat by œstrogen injections. We have personally confirmed the results of Hohlweg. We are therefore convinced that the pituitary releases LH under the influence of œstrogen. Does this mean

combined action of thiourea and œstrogens in male rats, normal and castrated. Our experiments included study of the weight of the thyroid; thyroid cell height and pituitary structure; the action of thyroxine; and we have tried to establish the quantities of thyroxine necessary to determine the regression of the thyroid in normal, castrated or œstrogen treated rats.

Table III

INFLUENCE OF VARIOUS DOSAGES OF ŒSTROGEN COMBINED WITH THE SAME DOSAGE OF 25 MG. THIOURACIL DAILY ON BODY AND ORGAN WEIGHTS IN MALE RATS

Treatment administered	*Body weight in g*		*Weight of organs in mg.*			*Relative weight of thyroid for 225 g body weight*
	Beginning	*End*	*Hypophysis*	*Thyroid*	*Adrenals*	
Controls	182·5	203·5	6·9	24·0	31·9	30·5±0·6
Propylthiouracil	195·5	172	7·5	48·7	31·2	56·2±3·2
Œstradiol benz 200 µg	190·5	190	21·3	27	48·5	32·6±0·12
Propylthiouracil + œstradiol benz 200 µg	187·5	146	17·6	43·6	37·8	52·6±2·4
Œstradiol benz. 50 µg	183	182	18·1	26·3	46·2	36 ±1
Propylthiouracil + œstradiol benz. 50 µg	187	147·5	15·4	40·6	38·3	49·2±3·3
Œstradiol benz 5 µg	190·5	197	14	25·9	40	30·6
Propylthiouracil + œstradiol benz. 5 µg	193	157	14·5	45·3	39·6	53·7±1·8

We could find that in accordance with the results of Severinghaus *et al.*; Nelson and Hickman; Clarke; Leblond, Albert and Selye, in the thyroidectomized animal, the administration of œstrogen could completely inhibit the appearance of the thyroidectomy cells in rats treated with thiouracil. Nevertheless, the thyrotrophic activity of the pituitary was not in the least influenced by this treatment and the weight and cellular height of the epithelium of the thyroid were not reduced in these animals. These results are shown in Table II and for various dosages of œstrogen in Table III.

between these elements (Zeckwer). The first opinion was supported by the fact that the administration of œstrogen may completely inhibit the appearance of the so-called thyroidectomy cells.

The use of the thyro-inhibitors of the thiourea group showed that this same pituitary picture may be obtained without removal of the thyroid by inhibition of thyroxine

Table II

Mean Weights and Mean Height of the Epithelium of the Thyroid in Normal or Castrated Male Rats Treated with Thiouracil or with Œstradiol Benzoate and Thiouracil

	Mean weight of the thyroid in mg	*Height of epithelium in μ*
Normal males	28 2±1 19	9 01±0 17
Castrated males one month after castration	24·7±0 97	
Normal males treated with 5 μg œstradiol benzoate daily for one month	41 7±2 81	11 85±0 60
Normal males+25 mg. thiouracil daily in the food for 14 days	63 3±3 4	16 76±0 18
Castrated males one month after castration+thiouracil for 14 days	70 0±4 6	
Normal males treated with œstradiol benzoate 5 μg daily for one month +thiouracil for 14 days	76 1±5 4	20 87±1 5

The weights of the thyroids are relative weights and calculated for a constant body weight of 225 g.

The figures for cellular height result from measurement on 120 vesicles in every animal.

synthesis. It showed also that this picture and the paradoxical stimulation of the thyroid which result from the administration of thiourea regress by injecting thyroxine. This study led to the conclusion that the hypertrophied basophils are the site of elaboration of the pituitary thyroid stimulating hormone (Griesbach and Purves).

I have in the last few years, with the collaboration of A. M. Ermans, undertaken extensive experiments on the

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The administration of thyroxine to thiouracil-treated castrated animals showed regression of the thyroidectomy cells but not of the castration cells. It showed also that the dose of thyroxine necessary to bring about the correction of the thyroid weight in the thiouracil-treated rat is definitely greater than in the animal similarly treated but not submitted to œstrogen treatment (cf. Table IV).

Table IV

INFLUENCE OF INCREASING DOSAGES OF THYROXINE ON THE WEIGHT OF NORMAL, CASTRATED OR ŒSTROGEN TREATED (5 μG ŒSTRADIOL BENZOATE DAILY) MALE RATS ADMINISTERED THIOURACIL IN FOOD

	← Thiouracil → Dosage of thyroxine				Controls (no thiouracil)
	0	2 μg	3 μg	4 μg	
Normal male rats	55 6	54	47 1	30 6	31·5
Castrated .	66 6	50 8	25 2		
Œstradiol treated	64 4	40 2	58 8	42 4	

These experiments show that the thyroidectomy cells may disappear under the influence of œstrogen. Œstradiol benzoate is able to influence them as well as the castration cells. These two elements nevertheless react differently to thyroxine which does not inhibit the appearance of the castration cells. *The thyrotrophic activity of the hypophysis* of the animal submitted to the influence of thiouracil is not decreased if their basophils have regressed under the influence of œstrogen. This fact seems to cast some doubt on the origin of the thyrotrophic hormone in the basophils.

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STRUCTURAL CHANGES IN THE ANTERIOR PITUITARY WITH SPECIAL REFERENCE TO THE ADRENAL CORTEX

H. TUCHMANN-DUPLESSIS

THE existence of a functional relationship between the hypophysis and the adrenal cortex has for a long time been suspected by clinicians. If it has been possible to determine the control of adrenal cortical secretion by the adenohypophysis through the work of P. S. Smith, and more recently by the chemical isolation of the specific factor responsible for this control, the adrenocorticotrophic hormone (ACTH), the influence of the adrenal cortex on the function of the adenohypophysis is by no means so well determined.

It is on this particular problem of the inter-relationship between the adrenal cortex and the adenohypophysis that I wish to speak now. We believe that cytological examination is capable of revealing some of the secrets of the function of the anterior pituitary gland.

Methods

We have used white rats of the Wistar strain, of between 120 and 150 gm. body weight. In each group of rats we have examined the hypophyses of 12, eight in treated animals and four controls. The animals have been killed by coal gas and the hypophyses removed immediately to be fixed for 30 minutes in Helly's fluid, they have then been embedded in paraffin or in celloidin, and sections stained according to the technique which we have described elsewhere (Tuchmann-Duplessis, 1947).

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treated [illegible]
Autopsy is carried out 96 hours after the beginning of treatment. The adrenal glands are weighed and then fixed in 10 per cent formalin and examined histologically after staining with either Sudan III, or BZL Blue.

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DISCUSSION

EVERETT. In the œstrogen-treated ear graft animals what was the time interval between the injection of œstrogen and the occurrence of corpora lutea?

DESCLIN. The interval between the injection of œstrogen and sacri-

DESCLIN. We injected progesterone twice, and we did it according to the scheme you used in constant œstrus females, because in the male you cannot know about the œstrogenic activity of the ovary or about the cycle. In this graft there were many follicles which contained blood, but we did not see new-growing follicles. Of course, the germinal epithelium is not conserved, and there are many follicle cysts. The important fact is that after progesterone treatment we get corpora lutea in the ovary grafted in the ear, whereas these formations are always absent in the untreated male.

RAWSON: Did you assay the pituitaries of your animals receiving thiouracil for thyrotrophic hormone?

DESCLIN. No. We did not.

RAWSON: I would like to add that in the pituitaries of rats maintained on thiouracil we have been unable to demonstrate thyrotrophic hormone by a microhistometric method of assay in the chick. However, if we administer thyroxine, 15–20 μg. a day, we restore it to a normal level. We have also tested tetrabromthyronine, and have observed that about 15 times as much of that agent is required to restore the normal level of TSH in the pituitary of rats receiving thiouracil

pituitaries that have been converted to a normal histological state after the administration of thiouracil plus œstrogens contain thyrotrophic hormone.

number of eosinophil and basophil cells is reduced, and that of chromophobe cells increased. The eosinophil cells are less granular, and with this loss show alterations in shape.

The basophil cells are the site of more marked changes, consisting of loss of granulation and vacuolization of the cytoplasm, which is also only feebly stained. But there remain always a few normal, very active cells in addition to those with vacuolization and hyaline degeneration.

According to their relative abundance, the basophil cells of the rat after adrenalectomy can be arranged in three classes: those with either a few remaining granules or none at all, returning to the chromophobe state; those normal and very active; and those vacuolated and dying. As a result of the loss of granulation of the eosinophil and basophil cells, the chromophobe cell count is increased.

The description just made applies to the majority of rat hypophyses after adrenalectomy. Nevertheless, in some animals we have found several islets consisting of 10–15 granular bodies staining feebly basophil. It is likely that it is such bodies that have led certain authors to describe the hypophysis after adrenalectomy as being basophil.

The reduction in size and number of the chromophil cells and their marked loss of granulation testify to the predominance of excretory over secretory activity.

(b) Deoxycorticosterone Acetate (DCA)

The anterior pituitary lobe in animals treated with DCA is characterized by an intense chromophil appearance. The chromophil staining is very largely due to the increase in basophil cells. The eosinophils contain a richly granular material, the proportion of larger eosinophil cells is higher than normal, whilst the total number of eosinophils is normal or only slightly increased.

The basophil cells are much enlarged, and their cytoplasm contains numerous granulations. The nucleus is rounded, often moved to one side of the cell, and separated by a narrow band of cytoplasm from the apparatus of Golgi, which is

The following operations have been performed:—

(a) *Adrenalectomy:* The removal of the adrenals has been made at one operation; the animals have been maintained on a normal diet without addition of salt and sacrificed 8–10 days after operation.

(b) *Administration of deoxycorticosterone acetate:* One group received over a period of 18 days an injection of 5 mg. of deoxycorticosterone in oily solution every three days, to a total of 30 mg. per animal. The second group has been sacrificed 10 days after having been given a subcutaneous implantation of two tablets each of 10 mg. of deoxycorticosterone acetate.

(c) *Compound S of Reichstein* has been administered subcutaneously in amounts of 3 mg. each day for 10 days.

(d) *Cortisone acetate:* One group of rats was given 5 mg. each day for six days, and a second group the same dose for each of 12 days, that is to say a total of 30 and 60 mg. of cortisone for each animal.

(e) *ACTH* Animals were divided into three groups· the first was given 0 40 mg. over a period of 30 days, the second 1 mg. for 10 days, and the third 2 mg. for five days, the injections were made twice a day with an interval of eight hours between them.

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[illegible] mg. for 12 days.

Morphological Changes

Before giving the results of our observations, I think it would be useful to remind ourselves of the morphological criteria which permit us to judge of the functional state of the adenohypophysis. They are four in number: the numerical proportions between the chromophobe and chromophil cells, cellular shape, the abundance of granulations, and the appearance of the cytoplasmic structures, Golgi's apparatus and the mitochondria

In the intact animal, the numerical proportions of the three different kinds of cell, chromophobe, basophil and eosinophil, are relatively constant. Increased productive activity is shown usually by an increase in number and size of the chromophil cells. Conversely, decreased secretory activity or predominance of excretory activity are revealed by a reduction in the ratio of the number of chromophil to chromophobe cells Further, the cytoplasmic granulations which [illegible]

(a) Adrenalectomy

After the removal of the adrenals, the anterior lobe of the pituitary assumes a mainly chromophobe appearance. The

accentuated and many of the cells have completely lost their granulations. Neither vacuolization nor degeneration is seen. The eosinophil cells show slight loss of granulation. The number of chromophobe cells is slightly increased in proportion to the basophils. In no group have we observed a basophil reaction, nor the changes described by Crooke as reported in man by Golden *et al.* (1950).

(f) Thyroxine

The changes produced by the administration of thyroid hormone are already well known. They consist of chromophil accentuation with increase in the number and in the size of both eosinophils and basophils. The eosinophils often show evidence of loss of granulation. The basophils are hypertrophied but only feebly granulated; a certain number of them contain chromophobe vacuoles; small basophil cells strongly granular have practically disappeared completely.

(g) Œstrogens

Administration of œstrogens leads to an increase in weight of the hypophysis and a turning towards chromophobia in the anterior lobe.

With small doses one notices first a slight increase in the number and in the size of the basophil cells. This transitory hypertrophy of the basophils is followed rapidly by a progressive loss of granulation which is evidence of an intense excretory activity by these cells. With large doses, loss of granulation in the basophils becomes extremely important, so much so that after eight days of treatment one can only find a few basophils with normal granulation. The eosinophils, which early in treatment show signs of increased activity with hypertrophy of Golgi's apparatus, later suffer equally from loss of granulation, but this is always less marked than in the case of the basophils. There always remains a considerable number of eosinophils; their appearance resembles the "cells of pregnancy." The gland also shows a vasodilatation of importance. One can often see

hypertrophied. Small basophils, characteristic of young cells, are rare. Many of the hypertrophied basophil cells contain fine chromophobe vacuoles and show the hyaline changes described by Crooke (1935) in Cushing's syndrome.

The increase in number and the hypertrophy of the basophil cells in animals treated with DCA testify to the predominance of productive and accumulative over excretory activity.

(c) Compound S of Reichstein

The anterior lobe changes after administration of this compound are of the same kind as after treatment with DCA. The number of large basophil cells with clear cytoplasm and hypertrophied Golgi's apparatus is often even more increased than after DCA.

(d) Cortisone

The hypophysis is both hypertrophied and chromophil as after the administration of the two previous compounds. There are, however, some differences in appearance for the majority of the basophil cells are of average size and well granulated. The large basophils with hypertrophied apparatus of Golgi are less frequent, and only exceptionally can one find cells showing hyaline degeneration of their cytoplasm.

The eosinophils are well granulated and present a normal appearance. Chromophobe cells are diminished in number by comparison with controls.

(e) Adrenocorticotrophic Hormone (ACTH)

In the group of rats treated by small doses over 30 days, the changes in the hypophysis are very slight. The proportions of the different cells remain normal. Nevertheless, if the eosinophils appear normal from all points of view the size of the basophil cells is usually reduced. Large basophil cells are rare and their staining is feebler indicating reduced activity. In the group of rats which have been given strong doses of ACTH for five to ten days, the number of basophil cells is indisputably diminished. Loss of staining is more

Table II

BIOLOGICAL ASSAY OF THE ACTH CONTENT OF HYPOPHYSES OF FEMALE RATS "REPAIR TEST"

Group	*Weight of adrenal in mg/100 g body weight*	*Weight increase in percentage compared with controls*	*Percentage variation compared with animals treated with normal hypoph. gland*
Hypophysec. controls	9 0	—	—
Hypophysec + normal hypoph. gland	11 2	+24 4	—
Hypophysec. + cortisone Group I	16 1	+78 8	+43 7
Hypophysec. + cortisone Group II	17	+88 8	+51·7

According to the results shown in Table II, cortisone increases the ACTH content of the gland by 40–50 per cent. This increase in ACTH is accompanied by atrophy of the adrenal cortex, which is evidence of a failure of hypophyseal excretion of ACTH.

Substances which stimulate the activity of the adrenal cortex depress that of the adenohypophysis. They increase the excretion of ACTH and lower its production.

The results are particularly striking in the rats treated with Fenocycline. In our experiments the increase in weight of the adrenals in these animals was of the order of 80–100 per cent. Hypertrophy of the adrenal cortex was always more marked in the male than in the female rats. The results of the biological assays were equally instructive. On the basis of the "Repair Test," the ACTH content of the hypophyses of rats treated with Fenocycline was lowered by 70–80 per cent. This was a more marked fall than that found in the animals previously adrenalectomized.

Examination of Table III which summarizes the results of our observations, shows that agents which inhibit the activity of the adrenal cortex, such as DCA, Compound S of Reichstein and cortisone, increase the adrenocorticotrophic

in the neighbourhood of the dilated capillaries masses of blue colloid which perhaps represents remains of basophil cells. The cytological appearance in these hypophyses reflects an increase in the excretory activity of the basophil cells which can continue to their complete exhaustion.

Adrenocorticotrophic Content of the Adenohypophysis

The results of biological assays of the adrenocorticotrophic hormone content of hypophyseal glands are summarized in Tables I and II.

Table I

BIOLOGICAL ASSAY OF THE ACTH CONTENT OF HYPOPHYSES OF MALE RATS "REPAIR TEST"

Group	*Weight of adrenal in mg./100 g body weight*	*Weight increase in percentage compared with controls*	*Percentage variation compared with animals treated with normal hypoph gland*
Hypophysec. controls	8 8	—	—
Hypophysec +normal hypoph. gland	12 0	+36 3	—
Hypophysec +adrenalectomy Group I	10 2	+15 9	−17 6
Hypophysec +adrenalectomy Group II	9 8	+11 3	−22 4
Hypophysec +DCA Group I	14·8	+68·1	+23·3
Hypophysec. +DCA Group II	13 8	+53 5	+12 5

Examination of these results shows that the "degree of repair" of the adrenal cortex, which is a measure of the ACTH content of the hypophysis, varies with the nature of the preparation. The strongest stimulations of weight increase, 53–68 per cent, are obtained with hypophyses from rats previously treated with DCA. The smallest increases in weight, 11–16 per cent, are found when rats have been previously adrenalectomized. The hypophyses of the animals treated with DCA contain more ACTH than those of intact animals, whilst the ACTH content of the glands of rats previously adrenalectomized is less than the normal.

and decrease after adrenalectomy and after the administration of substances which stimulate the activity of the adrenal cortex. It is, therefore, reasonable to look for the origin of ACTH in these cells.

Among the experiments which we have tried, that which has been generally best studied is, without doubt, the effect of adrenalectomy. If all the authors agree in recognizing that after the removal of the adrenals the number of eosinophil cells decreases, their opinions vary somewhat in regard to the basophil cells, on which the evidence is less substantial. Nevertheless, with the exception of Colombo (1946), who found in the adrenalectomized rat an increase in number of basophils, the majority of authors report regressive changes in the basophils. Grollman and Firor (1935) in both dog and rat, Reese *et al.* (1939) in rat, report a constant decrease in the number and in the size of the basophils. The same is found in the work of authors who have studied the hypophysis in patients with Addison's disease Kraus (1927), Shumacker and Firor (1934), Crooke and Russell (1935) and Severinghaus (1938) believe that the most characteristic change to be found in the adenohypophysis in Addisonian patients is the diminution in number and the regressive changes in the basophils.

The functional capacity of the basophil cells in animals which have been adrenalectomized is variously estimated If some authors attribute to them increased function, others such as Reese *et al.* (1939), basing their comments on the appearance of Golgi's apparatus and the reduction in granulations, consider them to be hypo-active.

It appears to us that these estimations are in error. Under a single term, the authors in fact seem to confuse two distinct functions of the cell, its secretory and its excretory activities. It seems to us that the reduction in size of the cell, the loss of granulations and the atrophy of Golgi's apparatus in the basophil cells of the adrenalectomized animal are evidence of an increased excretory activity and not of a general loss of activity of the whole cell. This interpretation fits in well with the diminution in ACTH content of the hypophysis

activity of the hypophysis. Adrenalectomy and stimulations of adrenal cortical activity, such as by Fenocycline for example, on the contrary lower the adrenocorticotrophic activity of the hypophysis.

Table III

Preparation	*Cytological changes*			*ACTH content of hypophysis*	*State of adrenal cortex*
	Baso	*Eosino*	*Chromo*		
Adrenalectomy	−	−	+	−	0
DCA . .	+ +	− +	−	+	Slight atrophy
Compound S	+ +	− +	−	+	Slight atrophy
Cortisone	+	Normal	−	+	Marked atrophy
ACTH	−	Normal	+	not tested	Hypertrophy
Thyroxine	+	+	−	not tested	Hypertrophy
Œstrogens	− −	−	+ +	−	Hypertrophy

Discussion

It is quite certain that operations on the adrenal cortex modify the structure and the function of the anterior lobe of the pituitary gland. The coincidence found between the changes in the cytological appearance of the hypophysis and the variations in its content of ACTH raises two problems: that of the site of production of ACTH, and that of the influence of the adrenal cortex on the adenohypophysis.

In the absence of a specific histo-chemical test, the exact localization of the formation of ACTH can only be surmised indirectly, that is to say by examination of cytological data at the same time as the results of hormonal assays.

When one examines the results of morphological analysis, one fact stands out: the cells which are most frequently changed by experiments on the adrenal cortex are the basophil cells. They increase under the influence of corticosteroids

treated with corticosteroids has also been observed by Cheng and Sayers (1950). The authors state on the one hand that DCA increases the ACTH content of the gland, and on the other that DCA acts in opposition to the hormonal depletion which normally follows adrenalectomy.

Study of the hypophyses of animals treated with corticosteroids provides, therefore, an important argument in favour of the localization of corticotrophic function in the basophil cells. The rôle of the eosinophils being left for the moment out of consideration, increase in corticotrophic activity can be explained by quantitative increases in basophil cells.

In the course of our work, we have proved that there is a remarkable parallelism between the number of basophil cells and the ACTH content of the hypophysis. Hess and Hall (1951) have, however, announced the finding of a disappearance of chromophil cells in the hypophyses of rats treated with DCA. The conditions of their experiments were different from ours and it would be difficult to dispute their findings here.

Two other arguments can be found in favour of the participation of basophils in the elaboration of ACTH: the results of the administration both of ACTH, and of synthetic œstrogens. When ACTH is given in adequate dosage, it produces unmistakable involutionary changes in the basophils without affecting the eosinophils. If the number of basophils is not always markedly reduced and so has escaped several observations, there is no doubt about the constant finding of a reduction in the size of these cells and of their loss of granulation. Such changes, evidence of cellular hypofunction, have been detected both in prepuberal and adult rats by Koneff (1944).

On the other hand, in patients suffering from glomerulonephritis and treated with ACTH (400-500 mg.), Golden *et al.* (1950) have found an increase of 20–25 per cent in the number of basophil cells. However, it would seem unwise to place too much reliance on these findings which were obtained in two cases only dying from uræmia. It is known, in fact, that

found in the adrenalectomized animal, not only by us but also by other authors. Using the "Ascorbic acid test," Sayers and Cheng (1949) found that the ACTH content of the hypophysis of the adrenalectomized rat was lowered to as much as 80 per cent of the normal. Interventions such as traumatization and burns which also stimulate the adrenocorticotrophic activity of the hypophysis only lower its content of the hormone by 30 per cent.

The relationship found between the decrease in the number *of basophil cells and the lowering of the content of ACTH is,* without doubt, in favour of the theory that these cells take part in the elaboration of ACTH. This argument has only a relative value for an equal decrease in the number of eosinophils is also found in the hypophysis of the adrenalectomized animal. The importance of this latter finding has still to be explained.

Results obtained by the administration of corticosteroid seem to us to be more easily capable of interpretation. *Whether it is a question of DCA, of Compound S of Reichstein,* or of cortisone, the anterior lobe of the pituitary reacts with *the same chromophil activity in all three cases. If the eosino-*phil cells are only slightly or not at all changed, the increase in the number and size of the basophils is considerable. The hypertrophy of the cells, the abundance of granular material and the frequent vacuolization suggests that the secretory activity of the basophils is increased in comparison with their excretory function. One can imagine that the hormone being only slowly eliminated accumulates within the cell and ends by inhibiting its activity.

This interpretation appears to be confirmed by two facts: the atrophy of the adrenal cortex and the increase in corticotrophic potency of the hypophysis. If the atrophy of the adrenal is relatively slight after administration of DCA, or of *Compound S,* it is certainly unmistakable in animals treated with cortisone, and this rules out any doubt about the *inhibition of ACTH excretion by the hypophysis.* The increase in corticotrophic potency in the hypophyses of rats

treated with corticosteroids has also been observed by Cheng and Sayers (1950). The authors state on the one hand that DCA increases the ACTH content of the gland, and on the other that DCA acts in opposition to the hormonal depletion which normally follows adrenalectomy.

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uræmia is among the numerous factors which can provoke a basophil reaction in the hypophysis.

The specific effect on the basophil cells in rats treated with ACTH seems to us to assume an important significance. Many observations confirm that when an anterior lobe hormone is administered, the functional activity of the cells normally responsible for that hormone's production is inhibited.

The correlation between basophil cells and ACTH secretion is equally well demonstrated in animals treated with œstrogens. The basophils first show increased activity, followed very quickly by a marked loss in granulation. The cell literally empties itself of its contents and then disappears. Excretion of the product of the cell is accompanied by intense stimulation of the adrenal cortex, which leaves no doubt as to the nature of the hormonal content of the basophil cell. Besides, the disappearance of basophils coincides, as we have shown, with a marked fall in the corticotrophic potency of the hypophysis.

When the various observations are reviewed, one is inevitably driven to conclude that elaboration of ACTH is connected with the activity of the basophil cells. This hypothesis does not exclude the possibility of the basophils being concerned in the production of other hormones. The manner of the regulation of cellular activity is still unknown. It is possible, if not probable, that the adenohypophyseal cell can produce different products at different times.

The second problem raised by our observations is concerned with the functional relationships between the adrenal cortex and the adenohypophysis. They appear, subject to verification, to be similar to those which govern hypophyseal and gonadal relations. Adrenalectomy provokes not only a reduction in the number of chromophil cells but also a lowering of the ACTH content of the hypophysis. Everything occurs as if removal of the adrenals suppressed both the control which normally opposes liberation of the hormone and the stimulus which leads to its production. Administration of corticosteroids produces the reverse effects; they increase

the corticotrophic potency of the gland whilst at the same time probably inhibiting liberation of the hormone. Such stimulants of ACTH secretion as thyroxine and œstrogens seem to act by the same mechanism as does adrenalectomy.

Our interpretation, which is based on the ACTH content of the hypophysis, is open to several criticisms. If it is true that the hormonal content of a gland does not necessarily reflect its functional activity (it could be increased when the gland is at rest or inhibited, and reduced when it is suddenly activated), there are some indications that the factors which inhibit the adrenal cortex act in opposition to the liberation of ACTH. If this does not appear to be certain in the case of DCA, it is quite evident in the case of cortisone.

The action of cortisone is at first sight unexpected, particularly if one compares it with that of œstrogens on gonadotrophic function. Large doses of œstrogens produce, as is well known, a reduction in the number of chromophil cells in the hypophysis accompanied by a drop in the gonadotrophic potency. In the case of cortisone, on the contrary, the hypophysis continues to thrive and its hormonal content increases about 40–50 per cent. At the same time the liberation of ACTH is obviously reduced for the receptor organ, the adrenal cortex, undergoes atrophy.

The stimulating action of adrenalectomy on the excretion of ACTH appears to be supported by the observations of Taylor *et al.* (1949), on patients with Addison's disease. It was possible to detect appreciable quantities of ACTH in the blood of the patients who were not treated, that is to say those who had a reduced level of corticosteroids. On the contrary, in the treated patients with a higher level of corticosteroids, the authors were not able to detect the ACTH.

Results obtained by use of the stimulants of adrenal cortex, thyroxine and œstrogens, confirm this point of view. It seems as if an increase of cortical steroids inhibits the liberation of ACTH and leads to its accumulation in the cells of the hypophysis, whilst adrenalectomy and the administration of œstrogens have opposite effects.

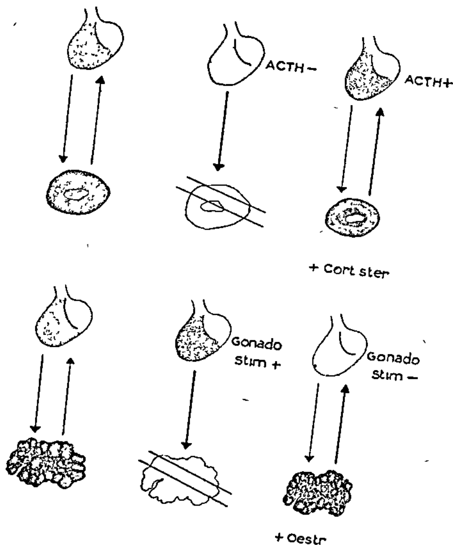


FIG. 1

As is seen in Fig. 1, the functional relationships between the adenohypophysis and adrenal cortex are different from those which exist between the hypophysis and the gonads. Whilst castration stimulates both liberation and production of gonadotrophins, adrenalectomy does not increase the elaboration of ACTH. In the former, the hormonal content of the gland is increased, in the latter it is reduced. There is a second difference in that the corticosteroids, whilst inhibiting liberation of ACTH, do not alter the cytological picture of the anterior lobe and do increase its hormonal content. Œstrogens, on the contrary, produce involutionary changes in the cells and reduce the hormonal content of the gland.

Although our observations suggest that quantitative variations in the levels of corticosteroids may play an important rôle in the mechanism of regulation of ACTH secretion, we do not propose to discuss this problem here, where it will be dealt with by more competent speakers. If our observations seem to fit in well with the theory of Sayers (1950), we should like just to mention a recent finding (Mercier and Tuchmann-Duplessis, 1951) which appears important to us. When one gives thyroxine to a rat, cellular metabolism is increased, and the hypophysis is stimulated and secretes increased quantities of ACTH which, in turn, produce hypertrophy of the adrenal cortex. By contrast, when one administers dinitrophenol, which is an equally potent stimulant of cellular metabolism, no hypertrophy of the adrenal cortex results. It seems, therefore, that

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[illegible]

involutionary changes in the hypophysis with reduction in its content of gonadotrophins

utilization by the tissues of corticosteroids may not be a sole factor in the regulation of ACTH secretion.

Summary

The influence of the adrenal cortex on the adenohypophysis of the rat has been studied by examinations of the cells of the hypophysis and of its hormonal content.

Seven groups of animals have been studied: (a) Adrenalectomized; (b) Treated with deoxycorticosterone acetate; (c) Treated with Compound S of Reichstein; (d) Treated with cortisone; (e) Treated with ACTH; (f) Treated with thyroxine and Fenocycline (œstrogen)

Removal of the adrenals produces a reduction in the number of eosinophil and basophil cells, and in the hormonal content of the gland. Under the influence of corticosteroids, the size and number of the basophils and also the hormonal content are increased. Fenocycline, which has been proved to have a powerful stimulatory effect on the adrenal cortex, produces a marked loss of granulations in the basophil cells and reduces considerably the hormonal content of the hypophysis. ACTH produces loss of granulations in the basophils.

The coincidence between changes in the basophil cells and in the hormonal content of the hypophysis indicates that the elaboration of ACTH takes place in the basophil cells.

Functional relationships between the hypophysis and the adrenal cortex appear to be different from those between the hypophysis and the gonads. Whilst removal of the gonads stimulates both production and liberation of the gonadotrophins, adrenalectomy only increases hormonal liberation. Administration of corticosteroids inhibits the liberation of ACTH and leads to its accumulation in the cells of the hypophysis.

The rôle of the adrenal cortex in the regulation of ACTH secretion is discussed.

[illegible]

and Fenocycline.

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DISCUSSION

HOUSSAY The relations between the adrenal and hypophysis are very complicated. There is an interrelation between both glands. After adrenalectomy in rats the secretion of pituitary adrenocorticotrophin is definitely increased We have seen that with Pinto (*Rev. Soc. argent. Biol*, 1944, 20, 38 and 108) in parabiosis experiments Normal female rats in parabiosis have an adrenal weight of 50 mg., but when one animal was adrenalectomized and was united in parabiosis with a normal animal the weight of the adrenals of the latter increased to 70–90 mg. or more We have also done adrenalectomy in pregnant rats, and we found that the adrenals of the new-born were

decreases after adrenalectomy The cytological aspect of the pituitary suggests that the ACTH decrease is related to an increased hormonal

output. According to this interpretation one may expect that the pituitary of an adrenalectomized animal would produce a greater stimulation to the adrenal in parabiotic experiments than the pituitary of an intact animal, as you find in your experiments It seems to me that adrenalectomy increases only the release of ACTH but not its production.

I agree with Professor Houssay concerning the adrenal-pituitary [illegible]

[illegible] only hormonal release (excretion), gonadectomy is followed by an increased hormonal production and increased release.

Dr. Sayers, who worked with the ascorbic acid test, found under the same conditions the same results as I did. He found that the decrease of the ACTH content in the rat 48 hours after adrenalectomy could be as great as 80 per cent. On the other hand, a few months later he found that if he gave DCA to an adrenalectomized rat the release of the ACTH of the pituitary was inhibited

[illegible] and cortisone [illegible] but our experience has been that there is no [illegible] guarantee that you have not ultimately given the animal 11-oxysteroids, because of the capacity of the adrenal very rapidly to introduce an oxygen in the 11-position.

TUCHMANN-DUPLESSIS: I agree entirely with Professor Long. The [illegible] quite different. If you give DCA you [illegible] while [illegible] erence [illegible] if you [illegible] self or compounds derived from DCA which act on the adrenals.

LONG: What is the mortality in your hands of total adrenalectomy?

TUCHMANN-DUPLESSIS: I kill them after 8–10 days because they die after about 10 days

LONG: Is there a possibility that some of those first changes that you observed may be due to a generalized adrenal cortical insufficiency, because they were dying then?

TUCHMANN-DUPLESSIS. I don't know.

HOUSSAY: In the strain of rats that have very severe insufficiency and die quickly, you will find the histological changes that you mentioned, but in the strain of rats that can survive, after a transitory and less severe insufficiency, you have much less histological change in the hypophysis.

Professor Houssay has observed.

HUME: With regard to Dr. Long's remark about not being able to get inhibition of the pituitary with deoxycorticosterone, we have used deoxycorticosterone glucoside, which has the advantage of not producing a significant eosinophil fall, and we find that we can inhibit pituitary activity with this substance in dogs. We have used large doses—60–75 mg. given intravenously. Furthermore, its effect doesn't seem to be on the basis of a conversion to Compound F or cortisone because minute amounts of Compound F produce a very good eosinophil fall in dogs, and these dogs have no significant eosinophil fall.

NELSON: I would like to refer for a moment to some experiments that Dr. Nich
yours I
into two
procedur
these cells very beautifully. The delta cells appear to be associated with thyrotrophin and with gonadotrophin, while it is the beta cell which shows changes under conditions of altered adrenal function, either adrenalectomy or administration of adrenocortical compounds. Although I couldn't distinguish, tinctorially, these cells on your photographs, I could do so by their shape, and it seemed to me that they were the cells that were undergoing changes. They are a smaller, more compressed cell than the delta cell.

TUCHMANN-DUPLESSIS: I didn't see his publication. I did the cyto-

different types of basophils. So I think that we are only entitled to draw conclusions if we find large changes in a great number of pituitaries. However, I am very glad to know that other people with more precise techniques came to the same conclusion.

PEARSE: I have endeavoured to use Halmi's not very easy technique

cells at all.

NELSON: But they do behave differently under these altered physiological conditi

PEARSE: Y
different stim
you want to,

NELSON: If

procedure, which really isn't difficult, you will find that on alteration of either the gonadal state or the thyroid state, the delta cells are the ones that undergo evident alteration; while under altered conditions of

PEARSE: Well, they're both the same cell.

NELSON. Dr Halmi doesn't think so

TIMIRAS There are many factors which can modify the response of the hypophysis and the adrenals to œstrogens, and I should like to consider two of them

One is the duration of œstrogen administration. When an adult rat is injected with œstrogens, it loses weight during the first two weeks of

Age is another factor which can influence the response of the hypo-

duration of the treatment does not affect the morphology of the adrenals in younger rats. However, in the immature as in the adult rat the

TUCHMANN-DUPLESSIS: For the tests with œstrogen we used two groups: one with high dosages and the other with very low dosages. With high doses the adrenal had a hypertrophy of 100 per cent. I think that after a treatment longer than ten days there may be repair of the pituitary, but we didn't do any experiments for longer than ten days. We only wanted to see if different agents which inhibit or stimulate the release of ACTH would cause some modification of the cytology of the pituitary.

JACOBSOHN May I refer to the parabiosis experiments again About ten years ago Westman and I did experiments on parabiotic rats (Westman, A., and Jacobsohn, D., 1944, *Acta path. microbiol. scand.*, Suppl LIV); we adrenalectomized one rat and hypophysectomized the other. We expected to get a good hypertrophy in the adrenals of the

hypophysectomized animals, but we were disappointed: there was no hypertrophy. We had at that time very few animals. Two years ago I used the same technique for a study of mammary gland growth (Jacobsohn, D., 1949, *Acta physiol. scand.*, 17, 423). A hypophysectomized female rat was joined in parabiosis with an adrenalectomized castrated female. The adrenals of the hypophysectomized mate were not hypertrophied as compared with normal glands. There was, however, an increase in adrenal gland weight, because if a non-adrenalectomized rat is joined to a hypophysectomized rat, adrenal atrophy occurs after hypophysectomy, and this atrophy is restored to normal (but not more) when the hypophysectomized rat is joined to an adrenalectomized mate.

TUCHMANN-DUPLESSIS: Do you think that if one removes the adrenals the secretion or the release of ACTH is increased or decreased?

JACOBSOHN: Increased.

TUCHMANN-DUPLESSIS. The excretion or the secretion?

JACOBSOHN. I observed these animals over a very long period, and the weight of the adrenals of the hypophysectomized partner remained normal during the whole time. I took out the hypophyses and examined them, and I couldn't find many basophils in the adrenalectomized castrated animals.

NERVE FIBRES IN THE RAT ADENOHYPOPHYSIS UNDER NORMAL AND EXPERIMENTAL CONDITIONS

E. VAZQUEZ-LOPEZ and P. C. WILLIAMS

Our attempt to describe in detail the innervation of the adenohypophysis is far from complete. The fragmentary evidence that we have is only worth presenting here because the question whether such innervation exists or not is so important in the controversy concerning the hypothalamic control of anterior pituitary function. We feel that any demonstration of nerve fibres in hitherto unsuspected locations and in profusion is important.

Material and Method

Description of the methods used is necessary not so much to explain our own positive results as the negative results of others. It is well known that the impregnation of nerve fibres is an uncertain procedure whatever method or material is employed. Some allowance for this uncertainty can be made by patiently repeating impregnation in a large number of glands, always bearing in mind that one positive result outweighs many negative ones, for in such a case the shortcomings of the method must always be suspected as the cause of the *failure of impregnation.*

With the pituitary gland, particularly of such a small animal as the rat, there is another difficulty. All silver methods applied to tissues in block produce super-impregnation of the surface areas so that study of these parts of the block is almost impossible. Where the tissue to be examined is as thin as the pituitary stalk the problem is difficult enough, but it is made even more so by the fact that the fibres we wish to demonstrate run on the very surface of

the stalk. It is easy to understand why ordinary methods fail in this purpose.

This surface superimpregnation can be minimized by using one of the variants of the Cajal method with minor modifications. Fixation for longer than normal in 10 per cent formalin plus 10–20 per cent chloral hydrate seems to reduce the argentophilia of the tissues. We have used tissues fixed for 10–30 days in this fixative and have found that the surface areas are then sufficiently resistant to impregnation to allow only the most argentophil tissues (that is the nerve fibres) to become impregnated. The later procedure follows the usual stages: incubation in 1·5 per cent silver nitrate for 5–6 days and reduction in pyrogallol and formalin.

Another way of improving the technical results of impregnation is to increase the argentophilia of the nerves. It is well known that nerve fibres are more easily stained under abnormal conditions and we (Vazquez-Lopez, 1949) have already taken advantage of this in studies of the nerve supply to the hypophysis in rabbits having spontaneous encephalitis—and have been criticized for it. We regard the criticism as ill-founded. The good impregnations obtained under such conditions are no more than particular cases of the general rule that degenerating and regenerating nerves have much greater silver affinity than normal ones; this basic, elementary fact of neurohistology sometimes seems to be forgotten. As the rat does not suffer from spontaneous encephalitis, we hoped to improve the impregnations by using partly or completely hypophysectomized rats. Examination of these at intervals after operation might also provide evidence about the survival and regeneration of nerve fibres in the different parts of the gland after injury.

Observations in Intact Rats

In normal rats we almost always find a thick bundle of nerve fibres running along the full extent of the pars tuberalis on the surface and very near the inferior aspect of the gland. The bundle is stained deep black and sharply contrasts with

the yellow or brown staining of the other structures unless the capsule of the organ is overstained when it is almost impossible to distinguish the bundle in the uniformly black superficial region. The course of the bundle is rather irregular, following caudally the general direction of the portal vessels and being very close to them in some places. It is impossible to follow its whole course in isolated sections which only show segments of it At the cephalic end of the pars tuberalis, almost at the level of the sulcus infundibularis, it appears as thin fascicles or as single fibres which are collected together caudally to form the bundle seen in Figs 1 and 2.

In connection with the vagaries of silver impregnations, it is worth examining this section in some detail. In most respects the impregnation is very successful and the two nerve tracts are clearly shown: the hypothalamic-hypophyseal tract in the upper region corresponding to the neural stalk, and the tract that eventually reaches the pars distalis in the lower region among the tissue of the pars tuberalis. The specificity of the impregnation is shown by the lack of staining both of the connective tissue surrounding the loops of the portal vessels and of the fibrous capsule which lies flat and partly detached near the lower border of the stalk. In spite of these excellent results it will be noticed that the marginal zone of the neural stalk appears absolutely devoid of nerve elements though it will be shown later that this zone actually contains a substantial number of fibres. This failure to demonstrate them is partly due to the difficulty of focusing them in this field, for under the microscope some fibres can be seen running vertically between the capillary loops, though their number is small. This typifies the difficulties of silver impregnation: almost perfect impregnation in the hypothalamic-hypophyseal tract and pars tuberalis and almost complete refractoriness in the tissue between, despite the very small area involved.

In serial or semi-serial sections it is possible to follow the complete course of the nerve bundle to the pars distalis where it enters that region of the gland, in the neighbourhood of the anterior hypophyseal artery (artery of the posterior



Fig. 1. Sagittal section of hypothalamus and median eminence of a normal rat showing the third ventricle (V), hypothalamic-hypophyseal tract (H), and bundles of nerve fibres (N) running along the pars tuberalis. (×80)

[illegible]

the yellow or brown staining of the other structures unless the capsule of the organ is overstained when it is almost impossible to distinguish the bundle in the uniformly black superficial region. The course of the bundle is rather irregular, following caudally the general direction of the portal vessels and being very close to them in some places. It is impossible to follow its whole course in isolated sections which only show segments of it. At the cephalic end of the pars tuberalis, almost at the level of the sulcus infundibularis, it appears as thin fascicles or as single fibres which are collected together caudally to form the bundle seen in Figs. 1 and 2.

In connection with the vagaries of silver impregnations, it is worth examining this section in some detail. In most respects the impregnation is very successful and the two nerve tracts are clearly shown: the hypothalamic-hypophyseal tract in the upper region corresponding to the neural stalk, and the tract that eventually reaches the pars distalis in the lower region among the tissue of the pars tuberalis. The specificity of the impregnation is shown by the lack of staining both of the connective tissue surrounding the loops of the portal vessels and of the fibrous capsule which lies flat and partly detached near the lower border of the stalk. In spite of these excellent results it will be noticed that the marginal zone of the neural stalk appears absolutely devoid of nerve elements though it will be shown later that this zone actually contains a substantial number of fibres This failure to demonstrate them is partly due to the difficulty of focusing them in this field, for under the microscope some fibres can be seen running vertically between the capillary loops, though their number is small. This typifies the difficulties of silver impregnation: almost perfect impregnation in the hypothalamic-hypophyseal tract and pars tuberalis and almost complete refractoriness in the tissue between, despite the very small area involved.

In serial or semi-serial sections it is possible to follow the complete course of the nerve bundle to the pars distalis where it enters that region of the gland, in the neighbourhood of the anterior hypophyseal artery (artery of the posterior



Fig. 5. A section nearer the sagittal (medial or axial) plane than that show
in Figs 3 and 4 More of the pars tuberalis is visible and the nerve fibres a
collected into thicker bundles Vessels of the portal system can be seen
the cephalic end of the median eminence and some tissue of the pars distal
(D) is present (× 80.)





FIG. 3 Para-medial section of hypothalamus from a normal rat only the most lateral segment of the pars tuberalis is visible Several fascicles of nerve fibres can be seen running over the glandular cells (80)

FIG 4. Enlargement of part of Fig. 3 showing the typical character of the nerve fibres (N) running among the cells of the pars tuberalis (T) The capsule of the gland and the connective tissue layer separating the brain from the pars tuberalis are brown in the section and easily distinguished from the black nerves. (×220.)



FIG. 9.

FIGS. 7–9. Semi-serial sections still nearer the sagittal plane than those shown in [illegible]



FIG. 10. Pituitary stalk (S) and surrounding area in a rat with a supernumerary pars nervosa (SPN). H indicates the hypothalamic-hypophyseal tract, I the infundibulum, and D the pars distalis. (×80)

Fig 7





Fig. 9.

Figs 7-9. Semi-serial sections still nearer the sagittal plane than those [illegible]



SPN

Fig. 10. Pituitary stalk (S) and surrounding area in a rat with a supernumerary pars nervosa (SPN). H indicates the hypothalamic-hypophyseal tract, I the infundibulum, and D the pars distalis. (×80.)



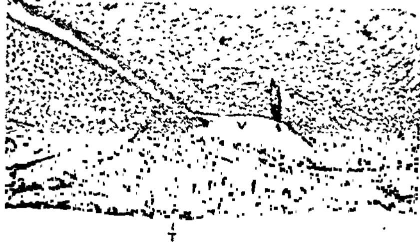


Fig. 13 Sagittal section of hypothalamus and median eminence of a rat 140 ... portal vessels ... vessels in the ... (× 80)



Fig. 14 Enlargement of ... from t' much nerve much

N

FIG 11 Enlargement of the supernumerary pars nervosa in another section, the break in continuity with the proximal section of the stalk is accidental. N indicates a fascicle of nerve fibres running towards the pars distalis and P the posterior extension of the stalk, lettering otherwise as in Fig 10 (× 165)

FIG 12 Further enlargement of another section of the supernumerary pars



Fig. 13 ...
days after ...
in the me...
directio...



Fig. 14 Enlargement of part of Fig. 13 showing the ...



FIG 15 A more proximal section than that in Fig 14 and without any portal vessels. The nerve fibres (N) run between the hypothalamic-hypophyseal tract (H) and the pars tuberalis (T). (×220)

lobe), which corresponds to the zona tuberalis of other mammals. However, we have never been able to trace it beyond this point, either as a bundle or as isolated fibres among the glandular cells.

The only advantage in the use of uncertain methods, such as those of silver impregnation, is that one is obliged to use a large number of animals to ensure a proportion of positive results. Thanks to this one often finds some cases differing somewhat from the normal and offering better conditions for observation. One such anomalous case is illustrated in Figs. 3–9, which are taken from an apparently normal male rat aged about 18 months. In this rat the bundle of nerve fibres running along the pars tuberalis is unusually thick and large so that in some sections it can be observed for the greater part of its course to its termination well inside the pars distalis. Fig. 3 shows a para-sagittal section of the hypothalamus with only the most lateral fragment of the pars tuberalis. Several fascicles of nerve fibres are clearly seen running over the glandular cells. A section nearer the sagittal plane is illustrated in Fig. 5, which shows a similar picture with the nerve fibres still divided into small segments and individual fascicles coming from different directions. The distinctive character of the component fibres can be seen in Figs. 4 and 6, taken at higher magnification. The individual fibres forming the fascicles can be seen in these pictures to have the typical morphological characteristics of nerve fibres. Although the impregnation of this specimen is fairly satisfactory, the monochrome reproduction of the microphotographs in black and white lessens the difference between the black of the nerve fibres and the various brown tones of the connective tissue in the capsule and borders of the brain, which is much clearer in the actual preparations.

Figs. 7–9 show further semi-serial sections in the more median planes of the same specimen; they show increasingly greater lengths of the bundle of fibres, which in Fig. 9 reaches well inside the pars distalis in the region of the anterior hypophyseal artery (artery of the posterior lobe). The bundle

runs along the whole length of the pars tuberalis, although the cephalic part near the sulcus infundibularis of the tuber cinereum and optic chiasma is not visible in the section. It comprises a very long segment exactly similar to a typical nerve trunk in structure, even showing the wave-like inflection common to small nerves along their course before their final ramification. In this animal, as in all the others we have studied, the impregnation of the fibres stops at the point where they enter the pars distalis proper and no isolated fibres or nerve endings are visible among the glandular cells. We assume that this nerve-like structure has the pars distalis as its destination although the final stages of the component nerve fibres still remain to be discovered.

There are two main possibilities regarding the origin of these fibres: that they originate in the hypothalamic nuclei and reach the pars tuberalis via the hypothalamic-hypophyseal tract or that they originate in the cervical sympathetic ganglia and reach the pars tuberalis together with the blood vessels forming the portal system of the pituitary stalk.

We have not yet examined the pituitary gland from sympathectomized rats but intend to do so as soon as possible. Until this is done we cannot dismiss the possibility of the sympathetic origin of the nerve fibres in the pars tuberalis, though Westman, Jacobsohn and Hillarp (1943) have described the persistence of a similar system of fibres after sympathectomy in the rabbit.

There is some evidence favouring the hypothalamic origin of the fibres—*in the literature concerning other species and in our own experience with the rat. The literature contains a number of reports of fibres leaving the hypothalamic-hypophyseal tract by turning sharply, almost at right angles, and vertically crossing the marginal or peripheral zone of the neural stalk to follow the direction of the portal vessels and* even to reach the pars tuberalis. Perivascular fibres in the marginal zone of the neural stalk have been described in the horse (Vazquez-Lopez, 1942) and cat (Nowakowski, 1951), and fibres with a similar course and a destination in the

glandular cells of the pars and zona tuberalis have been found in the rabbit (Vazquez-Lopez, 1949). They have been described even by authors who deny the existence of nerves in the adenohypophysis. Among the most recent work Rumbaur (1950) admits that one of these fibres could be traced from the hypothalamic-hypophyseal tract right into the pars tuberalis in the cat.

It is true that many of the fibres in the margins of the neural stalk and adjacent zones of the pars tuberalis end in the perivascular zone surrounding the capillary loops and portal vessels, and have terminal apparatuses exactly like those found in the rest of the neurohypophysis and, therefore, probably of a similar sensorial nature. Stutinsky (1948) has described nerve fibres of this character not only in connection with the blood vessels, but forming specialized receptor organs in the interstitial tissue of the pars tuberalis in the horse. Yet he also found other fibres of an excito-secretor nature reaching the colloid vesicles and the glandular tissue in the horse and also in the ox, sheep, pig and dog.

Our evidence for the hypothalamic origin of the nerve fibres in the pars tuberalis of the rat is derived from study of the rat which had the particularly large nerve bundle already fully described, as well as from observations in hypophysectomized rats to be described later. The large bundle illustrated in Figs. 8–9 was apparently confined to the one side of the stalk while the other side showed that irregular disposition of the neurohypophysis already described in the rabbit as a supernumerary pars nervosa (Vazquez-Lopez, 1949). This consists of an enlargement of the zone of the stalk adjacent to the zona tuberalis (or in this case to its homologue) leading to altered relations between the neurohypophysis and adenohypophysis. Normally the neural part is nowhere in direct contact with the glandular tissue of the pars distalis whereas in these cases there is a greater or lesser area of contact between the two divisions of the organ making it possible to follow nerve fibres passing from one to the other.

Fig. 10 is a general view of the anatomical relations among

the different divisions of the gland with a supernumerary pars nervosa. The alterations in the neural stalk consist of its enlargement in the direction of the pars distalis and the abnormal bulge projecting into the infundibular cavity (the missing tissue at the base of the gland in the zone of contact with the supernumerary pars nervosa is due to a technical fault). The hypothalamic-hypophyseal tract can be seen running past the supernumerary pars nervosa along its normal course, and the rest of the organ, including the pars nervosa proper, shows no abnormality. Higher magnification of another section (Fig. 11) shows fibres from the hypothalamic-hypophyseal tract passing diagonally to the supernumerary pars nervosa, and collecting caudally in a bundle of fibres which crosses the border of the pars distalis and which can be followed for a certain distance into the glandular tissue. The direction of this bundle within the pars distalis is, like that from the pars tuberalis described above, towards the zone where the anterior hypophyseal artery enters the gland. The even higher magnification in Fig. 12 shows the typical morphology of the nerve fibres coming from the hypothalamic-hypophyseal tract and joining the bundle passing towards the pars distalis.

Observations in Hypophysectomized Rats

This is a very incomplete study so we only mention the points that are immediately relevant to the origin and destination of the nerve fibres in the marginal zone of the neural stalk and pars tuberalis.

In the intact rat impregnation of nerve fibres passing perpendicularly from the hypothalamic-hypophyseal tract to the pars tuberalis is very rare or absent: in the partially hypophysectomized rat it is quite otherwise. The rats are those from which the body of the gland has been completely removed but in which the pars tuberalis remains intact and may even show hyperplasia. In such animals a considerable number of nerve fibres running in the hypothalamic-hypophyseal tract change direction to cross the marginal zone of

the neural stalk vertically in the free spaces between the loops of the portal vessels so as to reach the pars tuberalis. Here they revert to the horizontal and follow the same course over the glandular cells as the fascicles described in the intact rats. The number of these fibres varies from animal to animal and seems to increase with the interval after hypophysectomy though this point cannot be stressed without further investigation; it is nevertheless clear that the number is large in most hypophysectomized rats.

Fig. 13 shows a low-power magnification of the median eminence of a rat killed 140 days after hypophysectomy. The pituitary body has been completely removed but the pars tuberalis remains and the hypothalamic-hypophyseal tract is well preserved, especially in its proximal parts. The capillary loops of the portal vessels are perhaps more developed than in intact rats and in the spaces between them are isolated nerve fibres and bundles of fibres, which can be traced from the hypothalamic-hypophyseal tract into the pars tuberalis. Fig. 14 is a higher magnification of the central field of Fig. 13 showing the character of these fibres and their origin in the hypothalamic-hypophyseal tract. This is not a very specific impregnation, as some of the connective tissue is also impregnated, although in the actual section these connective-tissue tracts are brownish-yellow and easily distinguishable from the black nerve elements. Fig. 15 to some extent avoids this confusion. It shows a section from the same animal but of a region nearer the proximal end of the stalk where the vascular loops of the portal vessels are less numerous; fibres can be seen leaving the hypothalamic-hypophyseal tract to cross to the pars tuberalis where they resume their horizontal course.

There are two possible explanations of the many nerve fibres that can be demonstrated in the pars tuberalis of partially hypophysectomized rats. Either they are normal pre-existing fibres that become more easily stainable under the abnormal conditions or they are regenerating fibres following a new course. We cannot dismiss the second alternative mainly because the number of fibres seems to increase

with time after hypophysectomy, but even in this case it is probable that the new fibres will follow the course previously adopted by normal fibres. That normal fibres do take such a course, if only in small numbers, is probably because some of them have been seen in intact rats as well as in the other species listed above.

Very recently Stutinsky (1951) has studied these nerves in rats using the Gomori method. He has been able to follow fibres from the hypothalamic-hypophyseal tract to and through the pars tuberalis—but only in hypophysectomized rats. In intact rats they are invisible but the changed condition of the Gomori substance makes them clearly evident from the sixth day after hypophysectomy. This shows that in this region we have to take into account not only the alterations in staining properties that always exist in regenerating nerve but also of alterations due to changes in the peculiar substances that are normally present in the neurohypophysis.

For the present we believe that these observations establish the fact that regions of the pituitary stalk in which it is very difficult or impossible to demonstrate any nerve elements under normal conditions show a profusion of fibres under other conditions and that this can explain the origin of the nerve bundles running along the pars tuberalis in the direction of the pars distalis

Conclusions

It is obvious that the innervation of the rat adenohypophysis cannot be regarded as proved until the origin of the fibres we have described has been firmly established and their final course and endings have been described. Meanwhile we feel justified in drawing the following conclusions.

The impregnation of nerve fibres in the marginal zone of the neural stalk and pars tuberalis varies considerably according to the condition of the tissue.

In normal rats there are many nerve fibres forming bundles in the pars tuberalis running in the direction of the pars distalis.

The origin of these nerve fibres is uncertain but there is abundant evidence of fibres passing from the hypothalamic-hypophyseal tract to follow a course compatible with the hypothesis that these are the proximal segments.

More than three months after almost complete hypophysectomy, nerve fibres are demonstrable in great abundance in the remaining parts of the gland (pars tuberalis and neural stalk).

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DISCUSSION

NOWAKOWSKI I would like to ask if you are able to distinguish in the silver preparations neuroglial tissue and nerve fibres. Can you be sure that the fibres which run perpendicularly to the supraoptico-hypophyseal tract are really nerve fibres and not ependymal fibres?

VAZQUEZ-LOPEZ· I very rarely stain ependymal fibres with the Cajal method. They are not like the reticular fibres that I would expect to react. The ependymal fibres are more or less the prolongation of the [illegible]

VAZQUEZ-LOPEZ: No. We haven't done that but hope to do so soon.

HARRIS The problem that always comes up with these fibres is whether they are reticular fibres or whether they are nerve fibres The impression I got from some of the larger fibres in the slides that you showed was that they were very similar to the reticular fibres that are present around the trunks of the portal vessels in the pars tuberalis This is open, not so much to discussion, as to a simple experiment. Cut the pituitary stalk—if they are nerve fibres they will degenerate; if they are reticular fibres they will presumably remain there.

VAZQUEZ-LOPEZ· Of course, it is very evident that there is some connective tissue there But to believe that the nerve fibres and the

reticular fibres, when they are properly stained, cannot be distinguished, is a fallacy. There can be doubt in an isolated case or in some part of the fibre, but that is very exceptional. If you have reasonably good staining you have not only a bit of fibre, but some length of fibre. Of course, it is much easier to get good staining when you use frozen sections than when you use paraffin sections. There is no point in using paraffin sections for this kind of work because in very thin slices of tissue the nerve fibres go above and below the plane of the section, and you lose them. But when you get a fairly thick section like the frozen ones, you can follow the fibre up and down, although it makes it much more difficult to photograph.

HARRIS: But it would be rather more convincing to other people, I think, if you could produce two sections, one from a rat with the pituitary stalk cut, and one from a normal rat, mounted on a single slide and put through the same staining solutions in the same way.

VAZQUEZ-LOPEZ: That is why I showed you the second illustration. For there in the one section the nerve fibres of the hypothalamic-hypophyseal tract in the upper part and the fibres in the pars tuberalis in the lower part are perfectly stained, yet the fibres in between are

RICHARDSON: I should like to ask Dr. Vazquez-Lopez if he thinks it

which many histologists believe to be inevitable with some silver impregnation techniques, could be cleared up by detailed examination of individual sections treated in this way

VAZQUEZ-LOPEZ: Yes I am going to do that in a section containing the nerve tract. I expect I shall find plenty of reticular fibres there as well as in the anterior lobe

PEARSE: Would the nerve endings be adrenergic?

VAZQUEZ-LOPEZ: I expect they are.

GOMORI-POSITIVE AND GOMORI-NEGATIVE NERVE FIBRES IN THE NEUROHYPOPHYSIS AND THEIR PHYSIOLOGICAL SIGNIFICANCE

H. NOWAKOWSKI

WITH the aid of Gomori's method (chromhæmatoxilin-phloxin stain) Bargmann (1949) was able to stain electively the entire supraoptico-hypophyseal system in the cat and dog. It might be appropriate to consider the significance of this new staining method for some problems of hypophyseal physiology

Fig. 1 represents a paramedian saggital section through the hypophysis and hypothalamus of a cat stained with Gomori's chromhæmatoxilin-phloxin method.

The boundary between hypophysis and tuber cinereum is represented in all mammals by a macroscopically visible groove, the "sulcus infundibularis" (Spatz, Diepen and Gaupp, 1948). Proceeding from the proximal to the distal portion, three separate parts of the neurohypophysis can be distinguished histologically, corresponding to well-defined portions of the adenohypophysis:—

1 The "infundibulum" (=median eminence) with the "pars infundibularis of the adenohypophysis," the latter corresponding to Tilney's pars "tuberalis"

2. The hypophyseal stalk with a portion of the pars intermedia.

3. The posterior lobe, also bordering on the pars intermedia.

The adenohypophyseal tissue is always separated from the central nervous system (=tuber cinereum) by neurohypophyseal tissue. The neurohypophysis, in addition to its hormone-producing function, therefore must also have the function of a link between the adenohypophysis and the hypothalamus. With Spatz, Diepen and Gaupp we have

designated the zone of contact between adeno- and neuro-hypophyseal tissue as "adeno-neurohypophyseal contact surface" Certain findings indicate that a transport of substance takes place at this point.

Closer examination of the section shows that the supra-optico-hypophyseal tract is stained light-blue in its entire course from the nerve cells of the nucleus supraopticus, along the fibres through the infundibulum and hypophyseal stalk on to the terminal ramifications of the neurites in the posterior lobe. The Herring-bodies are stained in the same manner with an obviously intimate relationship to the nerve fibres. While the anterior lobe of the adenohypophysis is completely free of "Gomori-substance" the posterior lobe presents a surprisingly intensive blue reaction.

In a highly magnified frontal section through the supraoptic nucleus of the cat, stained in the same manner, we find in the cytoplasm of the nerve-cells an accumulation of Gomori-substance and dark blue fibres with globular engorgements: the neurites of the nerve-cells of the supraoptic nucleus. The blue substance along the entire course of the supraoptico-hypophyseal tract seems to be a specific product of the neurones. Bargmann believes that it is a secretion originating in the nucleus supraopticus and paraventricularis, and moving along the fibres towards the posterior lobe, where it is stored. These morphological findings are similar, however, to those observed by Cajal and later by Spatz (1921) in the central portions of the neurites following spinal cord transection. With Spatz we would therefore prefer to interpret these phenomena as a degenerative and not as a secretory phenomenon. In our opinion, a disintegration of the nerve substance takes place in the neurohypophysis under physiological conditions. If this assumption is correct, then logically there must be a process of regeneration proceeding *from the central stump of the axon. Regardless of the interpretation* given by these findings, they doubtlessly add strong support to the neurosecretion hypothesis of Gaupp and Scharrer (1935). Obviously the *fibres of the supraoptico-hypophyseal*

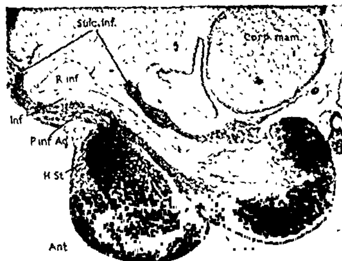


Fig. 1. Hypophysis and hypothalamus, adult cat. Chromhaematoxilin-phloxin stain. (22 ×.) Inf = infundibulum (= median eminence). H St = hypophyseal stalk; P L = posterior lobe, Sulc. inf. = sulcus infundibularis; R.inf. = recessus infundibularis, Corp. mam = corpus mamillare; P inf Ad = pars infundibularis of the adenohypophysis (= pars tuberalis). P int Ad = pars intermedia of the adenohypophysis, Ant.L. = anterior lobe.



Fig 2. Sagittal section through the ventral wall of the infundibulum (= median eminence). Silver impregnation of the nerve-fibres, Reumonts method (320 ×) R.inf. = recessus infundibularis; P inf.Ad = pars infundibularis of the adenohypophysis (= pars tuberalis); C.Z. = central zone; P.Z. = peripheral zone. Inf. = infundibulum (= median eminence).

Tub C.
R inf
Sulc. I
Inf. C Z
P Z.
P Inf. Ad.
P inf. A

Fig. 3 Infundibulum and tuber cinereum, frontal section, adult cat Chrom-hematoxilin-phloxin-stain Accumulation of Gomori-substance in the central zone of the infundibulum The boundary between the neurohypophyseal tissue and the tuber cinereum is marked by the "sulcus infundibularis" P inf Ad - pars infundibularis of the adenohypophysis, Inf = infundibulum (= median eminence), C Z = Central zone, P Z = peripheral zone, Sulc inf. = sulcus infundibularis, Tub.c = tuber cinereum



Fig. 4 ... nzidin-
stain (... Note
their clo... physis.
R.inf = ... nence);
P inf Ad ... eralis

tract not only conduct nervous impulses but *are directly concerned with the production of neurohypophyseal hormones.* This conception would explain the presence of the large number of nerve fibres in the neurohypophysis. Whether the Gomori-substance is the substrate of a neurohypophyseal hormone, a precursor or a prosthetic group is as yet uncertain. It seems therefore that the pituicytes are not as important in hormone production as we believed previously.

Perhaps the fact that the pituicytes belong to the glial tissue and may be satellite-cells of the nerve-fibres has not been considered sufficiently heretofore.

It is obvious that in the proximal parts of the neurohypophysis (infundibulum) the blue stained "Gomori-positive" fibre bundles of the supraoptico-hypophyseal tract are only located in the central zone of the infundibulum, whereas the peripheral zone—bordering on the "adeno-neurohypophyseal contact surface"—is nearly free of Gomori-substance.

A silver stain of this region (Fig. 2) shows that the fibre bundles of the supraoptico-hypophyseal tract pass through the central zone of the infundibulum. On the border of the peripheral zone a number of finer nerve fibres emerge at right angles toward the contact surface, forming a very delicate and rich network in the peripheral zone. This nerve fibre plexus is *not* stained by the Gomori method; in contrast to the blue-stained [illegible]
tract, this network [illegible]

In a frontal secti[illegible]
cinereum with a particularly clear representation of the morphology just described (Fig. 3), the contrast between the central zone and the peripheral zone of the infundibulum is striking. Now, what is the functional significance of the Gomori-*negative* nerve fibre system in the neurohypophysis, which we find only in the proximal parts? From the studies of Westman, Jacobsohn and Hillarp (1943), we know that these parts are of particular importance for the connection of the adenohypophysis, especially the anterior lobe, and the hypothalamus.

We find in the peripheral zone of the infundibulum peculiar vessels described by Green and Harris (1947) as "capillary loops," which I must discuss particularly, since we have made some definite deductions regarding their function, differing from the conception of Dr. Green and Dr. Harris.

In a frontal section through caudal parts of the infundibulum (Fig. 4) we can see that these infundibular vessels are isolated loops and intimately related to the capillary network of the adenohypophysis. Since they have no other afferent blood flow they must contain adeno hypophyseal blood conducted through the neurohypophyseal tissue in this manner. In our opinion (1951), these vessels are an expansion of the adeno-neurohypophyseal contact surface. An observation of Berblinger (1941) supports the view that adenohypophyseal blood circulates in these "special vessels": he was able to demonstrate the presence of FSH in the proximal parts of the neurohypophysis after removing the pars infundibularis (=pars "tuberalis"), not however in the posterior lobe. With this conclusion nothing is said concerning the direction of blood flow in the portal-vessels. In the immediate neighbourhood of these infundibular "special vessels," we find end divisions of our Gomori-negative nerve fibres. Whilst with reference to the anatomical findings there is extensive agreement with Dr. Green and Dr. Harris, we came to an exactly opposite interpretation of these findings. We believe that the hormones of the anterior lobe (e.g. FSH) circulating in the infundibular vessels stimulate the Gomori-negative nerve fibre plexus and that these impulses are conducted centripetally towards the parvo-cellular medioventral tuber cinereum ("sexual centre" of Bustamante, Spatz and Weisschedel, 1942). The direction of conduction of these fibres according to our conception would therefore be an *afferent* one, and not an *efferent* one. *The Gomori-negative fibres of the neurohypophysis we regard as dendrites of the parvocellular medioventral tuber cinereum, in contrast to the Gomori-positive neurites of the magnocellular supraoptico-hypophyseal system.* The Gomori-negative nerve fibres would therefore have a

chemoreceptory function responding to adenohypophyseal hormones. Vazquez-Lopez (1942) as well as Spatz, Diepen and Gaupp were the first to express the view that *neurohypophyseal nerve-fibres may have chemoreceptory functions.* According to this hypothesis, the medioventral tuber cinereum is consequently under direct control of the anterior lobe. An efferent regulation of anterior lobe function seems possible via sympathetic fibres, which doubtlessly enter the anterior lobe.

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DISCUSSION

HARRIS· I think there are some points that would be rather strongly against your view of the function of these vessels The slide you showed was of a cat. In other forms, like the rat, the primary plexus is far removed from the pars distalis So that if anterior pituitary hormones were circulated in the plexus which protrudes into the nervous tissue, the blood flow then would have to be from the pars distalis up the stalk towards the brain, which in fact it's not.

NOWAKOWSKI The capillaries protruding into the infundibulum of

in the adjacent adenohypophysis, in this case the pars infundibularis (=tuberalis). I have another question, how would you explain this observation of Berblinger that he found FSH in the proximal part of neurohypophysis?

HARRIS In which species was this?

NOWAKOWSKI· It was in cattle and in man, I believe.

HARRIS: I think you could explain that in many ways. It could be a matter of diffusion postmortem.

GREEN I would agree with Dr. Harris, because in some forms, for example, the turtle and the snake, the pars tuberalis is very small or absent, and the zone of contact is quite different, there is a connective

snakes

NOWAKOWSKI· No, I have only studied mammals.

VAZQUEZ-LOPEZ I might add that the nerves accompanying the portal vessels play a great part in innervation. The weight of evidence seems to be that they come down from the pars tuberalis to the anterior

things than hormonal precursors come down the stalk from the hypothalamus.

I should like to ask something about Gomori-positive and Gomori-negative staining. After hypophysectomy or section of the stalk, when the flow of Gomori substance is interrupted, don't you find Gomori staining everywhere, including the fibres I have described? The distinction between Gomori-positive and negative may not really be physiological

NOWAKOWSKI Following section of the stalk in the frog Hild found an accumulation of secretion at the site of injury. The proximal stump of the sectioned fibres was distended Similar experiments have not yet been done in mammals, so that it is not possible to conclude whether Gomori-positive substance is present in the peripheral zone of the infundibulum.

VAZQUEZ-LOPEZ· That may be possible in the stalk where nerve ... ng the Gomori ... In any case, ... nal zones, but

NOWAKOWSKI: I do not know the work of Stutinsky, but in my preparations I never found Gomori-positive nerve fibres in the pars infundibularis, and I believe this supports the idea that hypothalamic nerve fibres do not enter the adenohypophysis (except the pars intermedia).

substance.

SAWYER: I should like to question the importance of Berblinger's work to which Doctor Nowakowski referred, the finding of FSH in the

he tested with extracts of the hypothalamus.

COMPARATIVE ASPECTS OF THE HYPOPHYSIS, ESPECIALLY OF BLOOD SUPPLY AND INNERVATION

J. D. GREEN

MANY divergent views have been expressed about the interrelationship between the pituitary body and the nervous system. Some of these are summarized in Fig. 1. In view of these differences it seems reasonable to suppose that a morphological characteristic found in many divergent species is more likely to have functional importance than one which is found only in a few related animals. A comparative approach is of value from this point of view. Thus a brief recapitulation of some comparative aspects of the hypophysis may be desirable.

The terminology to be used is that previously proposed (Green, 1951*a*) and differs little from the usage of Rioch, Wislocki, and O'Leary (1940) except that it attempts to define more closely the limits of the various parts.

A true homologue of the pituitary has not been found in *Amphioxus lanceolatus*. A few observations on hemichordata (*Saccoglossus*) and urochordata (*Mogula*) have been equally inconclusive. Only adult forms have been studied in these animals· it is possible that the larval stages might show some signs of a pituitary gland.

In all species of animals so far investigated, from cyclostomes to man, the pituitary gland is subdivisible into an adenohypophyseal part developed from Rathke's pouch and a neurohypophyseal part developed from the diencephalon.

The cytology of the hypophysis is considerably more complex than was at one time thought to be the case. Whether these cytological appearances represent a variety of cell

types or a variety of stages of development, their study may well lead to an understanding of hypophyseal control.

Romeis (1940) has given detailed descriptions of many cells of the pars distalis, including two main basophil types and a variety of chromophobic and acidophilic cells. The two basophil types are best distinguished by the complicated Romeis procedure but they can also be shown by a simpler technique (Green, 1951*a*) and the latter may be usefully varied by combining it with the periodic-acid-Schiff reagent method of McManus. Besides these cells a number of hyperchromatic cells with pyknotic nuclei, both acidophils and basophils, are usually to be found in the pars distalis of the human pituitary.

Both basophil types are stained by Schiff's reagent but may be distinguished by the background colour of the cytoplasm. Certain large cells, presumably degranulated basophils, show irregular droplets, rods, crescents and annuli which stain strongly. Most of the colloid, including colloid in the pars tuberalis and hypophyseal vessels, stains deeply. A little fails to stain.

The periodic-acid-Schiff reaction is not specific for basophils. In acidophils occasional droplets are seen in the general vicinity of the Golgi net. Reticulum stains deeply and ground substance faintly. In the neural lobe the periodic-Schiff method shows basophils which have migrated into this region and there are also long spindle-shaped cells which show similar granules. These cells resemble the pigment cells so often reported in man. Similar cells may be found in the median eminence and neural stalk, especially in relation to the vessels.

The cytology of the neurohypophysis has been adequately discussed by Romeis (1940) and Harris (1948). Many cell types are found Curiously, there are more nuclei to be seen between the swathes of nerve fibres than in immediate relation to the vessels in the neural lobe. The term pituicyte is not specific. It includes many cells of different characteristics. Romeis (1940) has described these in detail. A curious

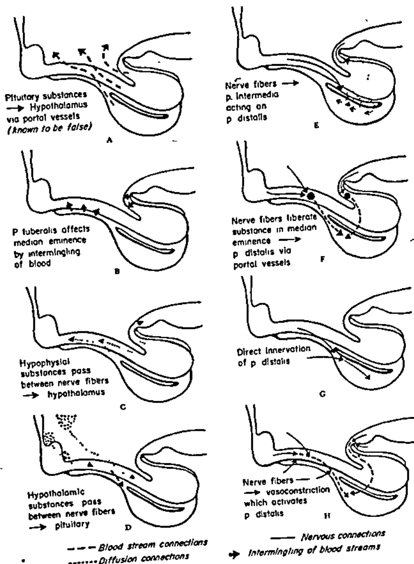

Pituitary substances
→ Hypothalamus
via portal vessels
(known to be false)
A
P tuberalis affects
median eminence
by intermingling
of blood
B
Hypophysial
substances pass
between nerve fibers
→ hypothalamus
C
Hypothalamic
substances pass
between nerve fibers
→ pituitary
D
Nerve fibers →
p. intermedia
acting on
p distalis
E
Nerve fibers liberate
substance in median
eminence →
p distalis via
portal vessels
F
Direct innervation
of p distalis
G
Nerve fibers —
→ vasoconstriction
which activates
p distalis
H
— — — Blood stream connections
.... Diffusion connections
——— Nervous connections
Intermingling of blood streams

FIG. 1.

protein-like substance pervades the neural lobe. It is readily stained by a variety of methods and may be seen by phase microscopy in preparations treated with phosphotungstic acid.

Collin (1937), the Scharrers (1945), Palay (1945), Bargmann (1949), and others have maintained that certain substances pass between the nerve fibres of the tractus hypophyseus either up the stalk to the hypothalamus or down it to the adenohypophysis. Intracellular "colloid" and material between nerve fibres can readily be seen, but the significance is dubious, for "secretory" granules may be seen in many unrelated parts of the central nervous system. In the hypothalamus and tegmentum, cells containing curious secretion-like granules can readily be stained by the periodic-acid-Schiff method.

FIG. 1 (*opposite*). Some relationships which have been suggested to occur between the nervous system and the adenohypophysis.

A variety of ideas has been expressed about the connections between the nervous system and the adenohypophysis. In this diagram an attempt is made to summarize some of these concepts and a few of the objections to them.

A Earliest concept of portal vessels of pituitary. Vessels believed to carry hormones to hypothalamus. Disproved by direct observation of direction of the blood flow.

B. Modified concept mingling of blood from pars tuberalis with that of median eminence. Cannot be universally applicable since some animals lack a pars tuberalis and others, for example Anurans, do not have the necessary vascular arrangement.

C and *D* Concepts of substances passing between the nerve fibres of the tractus hypophyseus, either up or down the stalk. Inconclusive evidence either for such transportation or for any activity of the material concerned.

F Although there is good experimental evidence for the production, in the median eminence, of a substance liberated by hypothalamic influences and activating gonadotrophin output by the pituitary, no such substance has so far been isolated.

G There are many objections, both on histological and physiological grounds for supposing a direct central or peripheral innervation of the pars distalis, though innervation of the pars tuberalis and pars intermedia is admitted.

H Control of pars distalis activity by a vasomotor mechanism would allow only a very crude regulation of hormone output.

The above brief note on cytology applies to mammals and particularly to man. In some lower forms, particularly in fishes, it is by no means certain that parallels may be drawn between the cells seen in their pituitaries and the pituitaries of higher animals.

The hypophysis of the lamprey consists of a flattened plate of glandular cells separated from the widely opened infundibulum by a fine vascular plexus. A few vessels pass into the adenohypophysis from this plexus. Throughout the vertebrates, a plexus lies between the adenohypophysis and the neurohypophysis. The arterial origin is constantly from the circle of Willis or its homologue and is more often derived from its anterior than its posterior parts.

In the fishes the adenohypophysis is found to be folded around a neurohypophysis which is very irregular in shape and shows many finger-like branchings. Despite the great specialization of almost every organ which occurs in the fishes, these relationships are constant. They are not affected by the morphological changes which occur with age (Woodman, 1939; Green, 1951*a*) nor by the wide species variations in the general shape of the gland. The vascular plexus may or may not penetrate the neural tissue, depending on species, and likewise may or may not communicate with the vessels of the saccus vasculosus. It consistently supplies the adenohypophysis and its venous effluent is constantly into perihypophyseal vessels.

Thus the neurohypophysis and adenohypophysis in fishes have a common blood supply which seems first to supply the nervous tissue and later the epithelial cells of the adenohypophysis. In the connective tissue between the two parts of the pituitary a certain amount of mingling of the two blood streams undoubtedly occurs, not only in fish but in most other animals. This could account, as Nowakowski (1951) has pointed out, for adenohypophyseal products acting on the neurohypophysis, but such actions still require to be proved. It seems much more likely that neurohypophyseal products act upon the pars distalis.

In amphibians the neurohypophysis receives blood from two sources: the median eminence is supplied with blood which then passes to the pars distalis. The neural lobe receives an independent blood supply. This independently vascularized region corresponds to the neural lobe in higher forms and it has been suggested (Green, 1951*a*) that this independence of vascularization provides the best means of distinguishing between the two parts of the gland. It is particularly interesting to note an apparent correlation between the size of this part of the gland and the animal's dependence on water in its environment (Green, 1947, 1951*a*). Thus a large neural lobe is found in the salamander *Plethodon cinereus* which is largely terrestrial, and a small one in amblystoma (chiefly aquatic). The neural lobe is likewise large in toads and small in frogs, large in snakes and lizards and small in turtles and alligators. The cetaceans apparently also have neural lobes which are small relative to the size of the rest of the gland.

Presumably, when animals first acquired a land habitat, the control of water loss from the body became of prime importance. The comparative physiology of the neurohypophyseal water-controlling factors has been thoroughly investigated, especially by Heller (1950) Surprisingly, it has been found that antidiuretic and amphibian water balance factors are present in substantial amounts in species from elasmobranch fishes to mammals. In fishes these substances have not been shown to have any influence on water balance but in higher forms their activity is pronounced. It has been found that in the kangaroo rat (Ames and Van Dyke, 1950), which lives in very dry surroundings, the concentration of antidiuretic substance is extremely high. The response of animals to water balance factors has also been shown to correlate with habitat to at least some extent.

The site of origin of these factors probably includes other regions besides the neural lobe. Sato (1928) and Van Dyke (1926) found significant concentrations in the neural stalk. Melville and Hare (1945) found rather large amounts in the supraoptic nucleus. Nevertheless, it seems that the greater

amount of these substances is produced in the neural lobe, and therefore the morphological changes observed may well be correlated with requirements for water.

Besides the appearance of a neural lobe in amphibians other remarkable changes are seen. The vessels to the pars distalis not only supply the median eminence and pars distalis but, in the anurans, they collect together into portal channels between the two vascular beds which may be called the primary and secondary capillary nets, even though the vessels of the pars distalis are more strictly sinusoids. A hypophyseo-portal system has been found first in the anurans and constantly thereafter in all the more advanced animals studied, its degree of development depending chiefly on the length of the hypophyseal stalk.

Sometimes the portal vessels are carried in specialized channels. Muller (1871) and von Economo (1899) described the "oberlappen" of birds which was supposed to transmit vessels from the dura to the pars distalis. One of von Economo's illustrations clearly shows the portal vessels passing from the median eminence *through* the dura. This arrangement is found not only in birds but in some reptiles (snakes and lizards) and in the whales. In these species the adenohypophysis is frequently separated from the brain by a septum of dura which is penetrated by the portal vessels and, apparently, by nothing else. This seems to be a strong argument for the importance of these vessels in hypophyseal control.

In more common animals (rabbit, cat, rhesus monkey, man) there is to be found a specialized region at the junction of the pars tuberalis, pars distalis and pars intermedia through which pass the largest trunks of the portal system. This has been called the "zona tuberalis" by Dawson and has also been regarded as a vascular hilum by earlier observers. The region is characterized by an abundance of connective tissue and rather small cells. It may be partially demarcated from the pars distalis proper by a connective tissue septum. Dawson (1948) has observed that the first cytological change produced

by a variety of endocrine interventions is in this area and that cytological changes follow the course of the portal vessels as they distribute themselves in the pars distalis.

The nature of the portal vessels in mammals is rather variable, but there are a number of common characteristics. The primary capillary net is common to the pars tuberalis and the median eminence and neural stalk. It consists of a dense plexus of fine vessels lying in the connective tissue septum between the two parts. From this plexus vessels penetrate the nervous tissue, particularly the cortical part external to the tractus hypophyseus, but also penetrating between the nerve fibres of the tract. In many species these vessels take the form of capillary loops, but in the Old World monkeys and in man they take the form of skein-like vessels. In most instances the vessels of the primary net are true capillaries, but in man and the Old World monkeys they have a more complicated histological structure, which has been previously described (Green, 1947). The arteries supplying the primary net for the most part enter as branches at the edge of the pars tuberalis. In man these branches are very numerous. The vessel walls show well defined elastic laminæ and the smooth muscle cells of the media are of the "epithelioid" type.

The structure of the portal vessels proper varies from simple, short sinusoidal vessels in animals with short hypophyseal stalks to well developed veins, as seen in the human hypophysis. The sinusoids of the secondary capillary net are of large diameter. They are surrounded by the curious connective tissue of the hypophysis which shows two elements: a fine one staining with the periodic acid-leucofuchsin technique and a coarser one staining with light green or aniline blue. Elastic tissue is not seen in their walls. This connective tissue surrounds both the sinusoids and groups of hypophyseal cells with a fine reticular net. Occasionally it appears to surround single large cells where these project from the side of a cluster. This reticulum may be shown in a variety of ways but is best seen in silver preparations or in phase-contrast preparations treated with phosphotungstic acid and mounted in a

medium of high refractive index. Pericellular nets were first described (as reticulum) by Tello (1912). Later, pericellular nerve endings were described (Pines, 1926; Brooks and Gersh, 1941; Hagen, 1950). The author has presented evidence from phase-contrast studies that reticular nets do exist (Green, 1951*b*). For this reason he is doubtful about the existence of nerve nets in the same situation.

Nerve fibres in the hypophysis are a particularly difficult problem. While it is impossible to demonstrate that nerve fibres do not exist in the pars distalis it is, on the other hand, necessary for the proponents of a direct secretory innervation to demonstrate clearly that any nerve fibres they find actually induce secretion. Histologists have wrangled for many years about this particular problem and on the differentiation of nerve fibres from reticulum in general (see Fig. 2). The issue depends entirely on the kind of evidence which the observer considers acceptable. In fine, no histological technique is specific for nerve fibres or endings. The best method seems to be that which can be best controlled.

A number of techniques have been tried. These have included methylene blue, various modifications of Cajal's and de Castro's techniques, Ranson's silver pyridine method, Bielchowsky methods and the techniques of Romanes, Reumont, Silver and Bodian. The latter, after slight modification, has been adopted as a routine because with it the most regular results were obtained. Observation of control areas of the same preparations showed an absence of visible reticular or collagen fibres but impregnation of fine nerve fibres in such situations as the molecular lamina of the cerebral cortex, sympathetic fibres accompanying blood vessels, pain endings in the pharyngeal mucous membrane and fine fibres accompanying blood vessels in the bone marrow. The impregnation of the neurohypophysis was detailed and nerve fibres were readily observed in the pars intermedia. Yet these preparations have consistently failed to show nerve fibres or endings in the pars distalis proper.

In preparations where glial or recticular fibres were visible

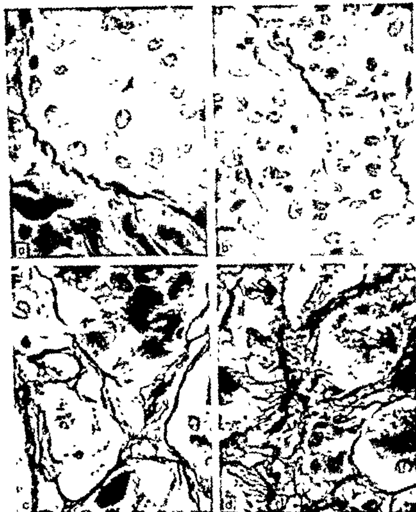


FIG. 2 Reticulum of adenohypophysis. All sections from adjacent regions in the same human gland. *A* and *B*—Gomori reticulum stain; inadequate staining due to faulty ammoniacal silver nitrate. Large collagen fibres are seen in *A*, and small fibres in *B*, which may be mistaken for nerves. In the other two photomicrographs the reticulum is more completely shown, but the correctly prepared stain has produced shrinkage artifacts.

[To face page 80



Fig 3 Bodian impregnations of the neurohypophysis of a Rhesus monkey. A—neural lobe At right the terminal part of the tractus hypophyseus is breaking up into the swathes of fibres, seen at the left, which surround blood vessels. B—frontal section of posterior part of median eminence and stalk. Note the tubero-hypophyseal tract, partially isolated by the plane of section. The use of fixation by injection reveals the primary

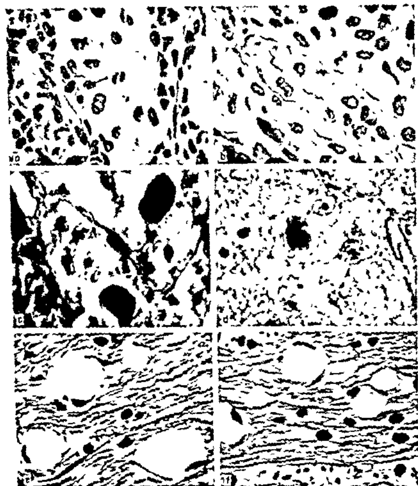


FIG. 4

A and *B* Nerve fibres in the pars intermedia of the Cebus monkey. *A*—rostral to the cleft, *B*—at edge of cleft

C Periodic acid-Schiff positive material (colloid) in the pars tuberalis (Human)

D P-A-S positive material in cells of the ventro-median nucleus (Human)

E and *F* "Osmoreceptors" in the internal capsule (E) and optic tract (F)



Fig 3 Bodian impregnations of the neurohypophysis of a Rhesus monkey. *A*—neural lobe At right the terminal part of the tractus hypophyseus is breaking up into the swathes of fibres, seen at the left, which surround blood vessels *B*—frontal [illegible]

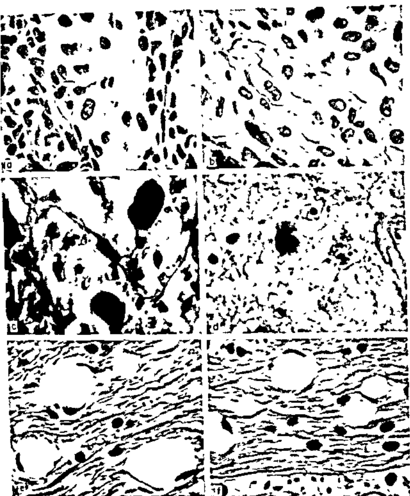


Fig. 4

A and *B*. Nerve fibres in the pars intermedia of the Cebus monkey. *A*—rostral to the cleft; *B*—at edge of cleft.

C. Periodic acid-Schiff positive material (colloid) in the pars tuberalis (Human).

D. P-A-S positive material in cells of the ventro-median nucleus (Human).

E and *F*. *"Osmoreceptors" in the internal capsule (E) and optic tract (F)*

in control regions (this is often the case with block techniques, modifications of the Bielchowsky method or Bodian preparations, where the gold chloride is too warm or partially exhausted) fibres were seen in the pars distalis which closely resembled nerve fibres. In other preparations from the same species a more detailed impregnation of nerve fibres in control regions and an absence of reticular or glial staining was obtained and in these no fibres whatsoever could be seen in detailed search of serial sections. Evidence that fibres resembling nerve fibres can be demonstrated by phase microscopy in sections in which nerve fibres in control regions are invisible also tends to support this thesis (Green, 1951*b*).

Nerve fibres are common in the pars tuberalis and pars intermedia. The former are derived both from the blood vessels and the tractus hypophyseus. They may extend along the portal vessels and occasionally reach the zona tuberalis. The pars intermedia derives its nerve fibres from the neural lobe. They often end in fork-shaped processes among the cells. A curious situation exists in the cebus monkey. Here the pars intermedia apparently is found both rostral and caudal to the hypophyseal cleft (see Fig. 4). At all events, cells of the same characteristic appearance are found on both sides. Fibres from the neural lobe enter the caudal part, sweep around the edges of the cleft and innervate the rostral part. The pars distalis proper shows no nerve fibres whatsoever.

The innervation of the neural lobe of mammals is generally accepted, even though there has been some dispute about the nature of the fibres seen there Some have contended that they are of a sensory nature. The evidence that the neural lobe is a secretory organ under the control of the nervous system (Fisher, Ingram and Ranson, 1938, Verney, 1948, Harris, 1948) makes it unlikely that *all* the nerve fibres are sensory, however. The source of these fibres is undoubtedly in part from the supraoptic nucleus and very probably from the closely related paraventricular nucleus also. It seems that the posterior part of the tractus hypophyseus is derived

from the lateral tuberal nuclei (Green, 1951*a*). A large number of fibres are also derived from the region ventral to the fornix in the anterior and lateral parts of the hypothalamus coming from the general region of the medial forebrain bundle (Fig. 3). Since these fibres disperse fanwise, their connections cannot be established. They may be of olfactory origin.

Recently "osmoreceptors" have been described in the supraoptic nucleus (Verney, 1948). Without in any way detracting from the excellent physiological work leading up to this conclusion, the histological evidence must be doubted. These vesicles may be seen in brains perfused at too high a pressure and probably represent dilated or ruptured capillaries (see Fig. 4). They are absent from specimens prepared by immersion fixation in which the brain has been carefully dissected from the base. Morin (1945) described similar structures produced by pulling on the leash of vessels which penetrates the supraoptic nucleus. He states that carefully perfused material failed to show them. Similar structures may be produced by perfusion at systolic blood pressure (or even less) in any part of the brain, particularly where the capillary bed is rich.

From a histologist's point of view it may be said that there is little sanction for direct secretory innervation of the pars distalis. If such innervation is lacking, then there must be either a humoral or vasomotor control of the gland under the control of the central nervous system. A humoral control could be exerted either through the systemic circulation or through the portal vessels and a hypothetical excitatory or inhibitory substance might be derived either from the median eminence proper or from the pars tuberalis which surrounds it. Extracts of this region have failed to induce ovulation (Westphal, 1949) or to yield adrenaline. Nevertheless, the work of Harris on hypophyseal stalk section (Harris, 1950) strongly suggests a humoral control through the portal vessels, at all events for gonadotrophin production. Furthermore, the arrangement of vessels found here may well allow of local action of a substance too weak to act when in

the general circulation or too rapidly destroyed by other tissues.

Adrenaline appears to induce responses of the kind which are rather arbitrarily collected together as the "stress" reaction. This may w
can apparei
to grafts in
et al., 1949) and since the perfused adrenal reacts to ACTH but not to adrenaline (Vogt, 1951). It is not impossible that more than one mechanism may be involved in this rather complex system.

Finally, the possibility of a vasomotor control of the hypophysis through the portal vessels may be considered. This, on the face of it, would appear an extremely crude mechanism. However, the hypophysis is a primitive organ; at least one vasomotor substance can apparently activate it and an active vasomotor substance is produced in the neurohypophysis. Furthermore, the immediate effect of cutting off the blood supply to the pars distalis in the toad is ovulation (Lascano-Gonzalez, 1935). It is rather fruitless to speculate in this superficial manner and without going into much greater detail; nevertheless, the control of the hypophysis cerebri is so complex that all possible mechanisms must be considered from a morphological as well as from an experimental standpoint. Since morphological interpretations are necessarily syllogistic it is important to consider very carefully the evidence on which they are based.

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DISCUSSION

BROBECK. This is something about which I've never found any clear statement in the literature. In animals that have these portal vessels, what proportion of the blood supply reaching the anterior lobe will pass through these vessels?

GREEN It depends on the species, I think. In the toads, I think Lascano-Gonzalez showed that if you cut off the supply by transecting the diencephalon, thereby interrupting the superior hypophyseal arteries, there was almost complete infarction of the pars distalis, except for a very small section posteriorly. In other animals there is a systemic supply, but my impression is that that is very largely capsular. I have no doubt that these vessels could supply the pars distalis in the event of an emergency, but my impression is that by far the majority of blood supplying the pars distalis is portal blood.

HOUSSAY: In 1935 Dr. Biasotti, Dr. Sammartino and I observed the hypophysis and the ventral surface of the hypothalamus and of the

anterior part of the hypophysis, but the pars intermedia, the neuro-hypophysis, and some parts of the anterior lobe are preserved because they also receive vessels from the brain and from the basilar artery.

hypophysis.

Green. This seems to be an extremely important point, and I have discussed it in a recent paper. One of the reasons that I suggested the possibility of a vasomotor control, even though it would be a crude mechanism, is this work in Dr. Houssay's laboratory.

Querido. I should like to ask Dr. Green if he has any idea about the magnitude of this portal circulation in human beings. And secondly, whether he has any ideas about the factors which are influencing it in, say, vasoconstriction-vasomotor reactions. I ask it for a special reason. I don't understand, first, why post-partum hæmorrhage leads to necrosis, and second, why large hæmorrhages in males never give these syndromes.

Green: I can only say that the portal circulation is extensive in the

in situ would probably recover as Dr. Houssay's frogs recovered (after about 30 days, I think). I suppose that a somatic blood supply could lead to recovery

very engorged or special endocrine changes may be involved I have no evidence one way or the other as to whether there is, in fact, an enlargement of the portal vessels during pregnancy.

CROOKE: I would entirely agree with you on the extent of the supply to the human pituitary. I have cut serial sections of about 800 human pituitaries. Admittedly I only examined every seventh section, but that took a couple of years, and out of that 300 I think I've only seen about half a dozen times minute vessels entering the capsule of the anterior lobe or anywhere other than vessels coming down the stalk. I think that practically the entire supply to the human pituitary must be by the stalk.

PART II

CONTROL OF SECRETIONS

THE RELATIONSHIP OF THE HYPOTHALAMUS TO THE PITUITARY SECRETION OF ACTH*

DAVID M. HUME

We have studied the effects of electrolytic lesions placed in various portions of the hypothalamus on the ability of the anterior pituitary to secrete ACTH after various stressing agents were applied. We have also studied the effects of remote control stimulation of the intact hypothalamus on pituitary ACTH secretion. We have tried to assess the effects of hypothalamic extracts on pituitary function. Brief abstracts of this work have already appeared (Hume, 1949; Hume and Wittenstein, 1950). Studies have been carried out on the rôle of the sympathetic and central nervous systems in [illegible]

this short note are still in progress and are incomplete as yet. All experiments have been carried out in dogs.

It has been shown that the fall in circulating eosinophils four hours after the injection of epinephrine is dependent on having an intact pituitary and adrenal cortex (Recant *et al.*, 1950). There is some question whether this is always so in the human (Thorn *et al.*, 1951), but it appears to be true for the dog. It is possible to take out the entire posterior lobe and most of the anterior lobe and still have a normal eosinopenia in response to injections of epinephrine. However, taking

*Supported by a grant in aid for research from the Commonwealth Fund.

out the entire anterior lobe completely abolishes the epinephrine-induced eosinopenia.

Effects of Hypothalamic Lesions on Eosinopenia induced by Epinephrine, Insulin, Mecholyl, and Operative Trauma

Certain lesions in the hypothalamus prevent the normally occurring eosinopenia following the injections of epinephrine, insulin and mecholyl. In two animals the eosinopenia following operative trauma was also abolished. These effects were always temporary, in that no animal continued to show this absence of response longer than six months after the lesion was made. It does not seem likely that post-operative œdema could explain this period of unresponsiveness for the following reasons: (1) In many cases the lack of response persisted for three to five months; (2) Very large lesions in some areas of the hypothalamus did not prevent the eosinopenic response at any time after they were made; and (3) Large lesions in the pituitary itself did not prevent the eosinopenic response following stressing agents.

The lesions which were effective in preventing eosinopenia following epinephrine, insulin, or mecholyl were located either in the anterior portion of the median eminence of the tuber cinereum, or, in two instances, in the posterior portion of the tuber and the anterior portion of the mammillary bodies. In the two animals in which the response to operative trauma was blocked as well, the location of the lesions were in the anterior portion of the median eminence. This area is shown diagrammatically in Fig. 1. Some involvement of the walls of the third ventricle at this level was also present.

The midline lesions in the floor of the third ventricle sometimes did and sometimes did not produce diabetes insipidus, depending on their lateral extent. The presence or absence of diabetes insipidus had no connection with the loss of the normal eosinopenic response following stress. Lesions made directly in the supra-optic nuclei, producing diabetes insipidus, had no effect whatsoever on the normal eosinopenic response

to stress. We have as yet no explanation for the observation that the animals begin to respond again after a long period of time.

The pre- and post-operative response of a typical animal with a hypothalamic lesion is shown in Fig. 2.

Section of the stalk along the plane shown by the dotted line in diagram 1 did not prevent the normal eosinopenic

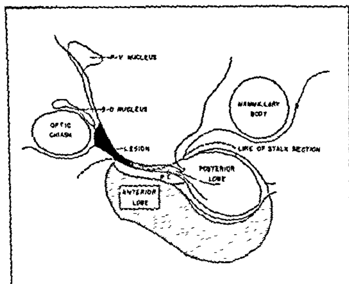


FIG 1 Diagram of sagittal section of hypothalamus and hypophysis of the dog to show the location of the lesions which appeared to be the most effective in abolishing the eosinopenic response to stress The third ventricle was dilated and its ependymal lining was damaged to varying extents at this level, also The dotted line illustrates the plane through which stalk section was carried out.

response following stress. Serial sections of the hypothalamus and the pituitary were made in these animals but indian ink injections of the blood vessels were not done. It cannot, therefore, be conclusively stated that the hypophyseal portal vessels had not regrown. However, one of the animals was tested only a week after the operation, and a polyethylene

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sheet had been placed between the pituitary and the hypothalamus after the stalk was severed, making it seem unlikely that the vessels could have re-formed in so short a time under these circumstances.

The Effects of Stimulation of the Hypothalamus in Intact and Totally Sympathectomized Animals

Electrodes attached to coils were implanted by a slight modification of the method used by Harris (1947), making it

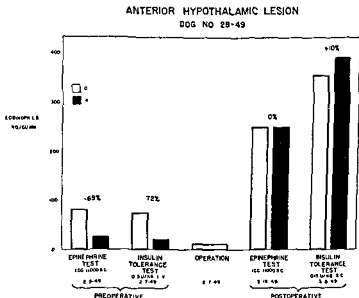
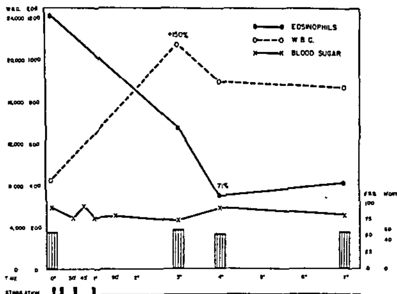


FIG. 2 The percentage fall in circulating eosinophils in response to epinephrine and insulin before and after a hypothalamic lesion Note the increase in the zero hour eosinophil count following the lesion

possible to do repeated remote control stimulations of the hypothalamus in the unanæsthetized dog. The effects of anterior hypothalamic stimulation in the intact dog are shown in Fig. 3. Four three-minute stimulation periods during the course of one hour were used. The current is a 60 cycle sine

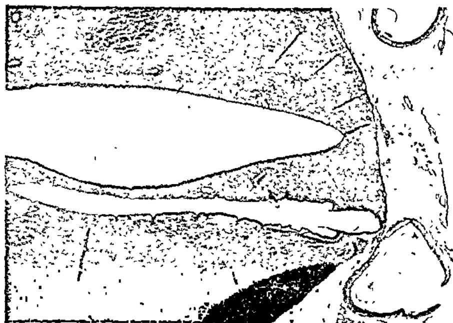
wave current, and this same effect can be produced by a single one-minute stimulation with 0·4 volts at 0·2 milliamps. The marked eosinopenia and the concomitant marked rise in total white blood cell count indicate that a release of ACTH has followed this hypothalamic stimulation. The stimulation

DOG NO 173 - COIL IMPLANT



response

is a very localized one and the dog gives no indication that he is aware of being stimulated. The location of the tip of the needle in this animal is shown by the photomicrograph in Fig. 4. It is just to the right of the midline in the floor of the hypothalamus posterior to the optic chiasma but anterior to the stalk.



To face page 211

seen that the marked eosinopenia and rise in white count following hypothalamic stimulation occur even without *release of endogenous epinephrine.*

To summarize the foregoing experiments: It was possible to make lesions in the hypothalamus which abolished the release of ACTH from the pituitary following stress although the pituitary itself was intact. Conversely, stimulation of the hypothalamus produced a release of ACTH from the pituitary even in the absence of the sympathetics. Section of the stalk did not appear to prevent the release of ACTH following stress. The development or absence of diabetes insipidus had no effect on ACTH release. We concluded from these experiments: (1) That the hypothalamus was important in the increased release of ACTH from the pituitary which occurs following stress, (2) That its effect on the pituitary need not be brought about by epinephrine or by pituitrin; and (3) That its effect must be mediated by some humoral substance because it was present even in the absence of any direct connection between the pituitary and the hypothalamus.

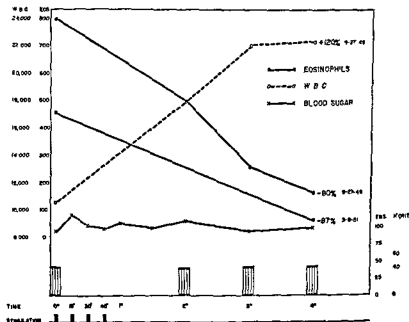
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It has been very difficult to assess the effects of hypothalamic extracts and to date we have obtained no consistent results with these substances. We are continuing to work with them.

The Effects of Section of the Spinal Cord on the Release of Pituitary ACTH Following Trauma to the Denervated Area

This is a difficult group of animals to work with and the results are not always consistent. There is some evidence that sympathetic, as well as somatic, afferents have to be eliminated, so that the limb is completely denervated, before one notes an absence of ACTH release following trauma to the denervated extremity. Fig. 6 shows the results of some

It is known that stimulation of the posterior hypothalamus leads to sympathetic excitation and thus to release of epinephrine from the adrenal medulla. Although we have not observed any evidences of epinephrine release following



totally sympathecto-

stimulation of the anterior hypothalamus, we had not completely ruled out epinephrine as the cause of the eosinopenia observed after hypothalamic stimulation. Therefore, coils were implanted in totally sympathectomized dogs. A diagram of the effects of hypothalamic stimulation in a totally sympathectomized dog is shown in Fig. 5. It may be

fall which was noted. A study is under way on sympathectomized dogs to check this point.*

Other Possible Mechanisms of ACTH Release

It is well known that epinephrine can produce a release of ACTH from the pituitary, and from the results of our experimentation it appears to us that this must be due to the effect of epinephrine on the hypothalamus rather than by a direct action on the pituitary, as postulated by McDermott *et al.* (1950). It has been shown above that the release of ACTH following hypothalamic stimulation does not depend on the presence of epinephrine. It was likewise shown, as may be seen in Fig. 7, by using a sympathectomized, unilaterally adrenalectomized, unilaterally adrenal-demedullectomized dog, that operative trauma produced a marked ACTH release even in the absence of epinephrine, as shown by the eosinopenia following this procedure. An injection of mecholyl likewise produced a marked eosinopenia in the absence of epinephrine. This response was abolished by injecting atrophine before injecting mecholyl. It has also been shown that the injection of insulin produces a marked eosinopenia in the absence of epinephrine.

An experiment was done to check the theory of Sayers (1947) that the traumatized area removes corticoids from the blood stream, thereby decreasing the amount of circulating corticoids and inducing ACTH release. A bilaterally adrenalectomized dog maintained on a DCA pellet was used for these experiments. An injection of Compound F was given intra-arterially and a dose was determined that was small enough to give just a significant fall of eosinophils (Fig. 8). The leg of this dog was now traumatized at operation, a procedure which of course produces no eosinopenia in an adrenalectomized dog, and the same amount of Compound F was now

*Subsequent experiments have shown that a sub-normal eosinopenic response can be produced by a severe burn below the level of cord section even after total sympathectomy This may be due to direct action of the products of tissue destruction upon the adrenal medulla or upon the hypothalamus

experiments on a dog with a cord section at the level of C7, and it will be noted that he no longer showed an eosinopenia following operative trauma to the denervated extremity, although he continued to show an eosinopenia following injections of epinephrine and to operative trauma above the

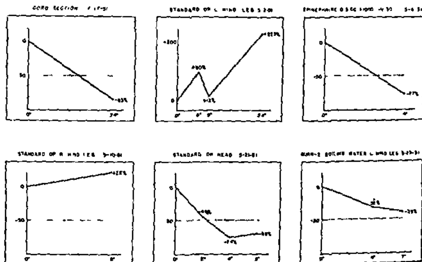


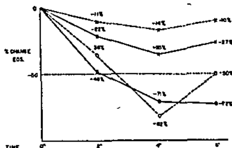
FIG. 6 The percentage change in eosinophils in response to trauma below and above the level of cord section The response to I-V epinephrine is also charted. No eosinopenia was induced by operative trauma to the denervated portion of the body, although a similar procedure done to the head produced a normal eosinopenic response. Severe trauma (burn) to the leg produced a minimal eosinopenic response, probably on the basis of reflex secretion of endogenous epinephrine.

level of cord section. Very severe burn trauma to the hind leg did produce a slight eosinopenia, though far less than one would see following such trauma in an intact animal. This animal was not sympathectomized and he developed marked mass reflexes when his leg was put in boiling water. This may have released enough endogenous epinephrine to produce the

area did not directly take Compound F from the blood stream. These experiments were carried out on a single adrenalectomized dog, and more animals will have to be studied to establish this point finally. The experiments cited above in

DOG NO 33-51 BILATERAL ADRENALECTOMY + DCA PELLET







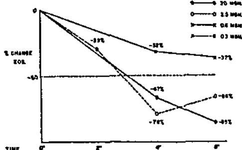


Fig. 8. [illegible]

which moderate trauma to the denervated extremity failed to produce a significant eosinopenia likewise suggest that direct utilization of corticoids by injured tissues was not the principal factor responsible for ACTH release under these circumstances.

injected intra-arterially so that it would come in contact with the traumatized area before gaining access to the general circulation. If the Compound F were removed from the blood stream by the traumatized area there should be less of it

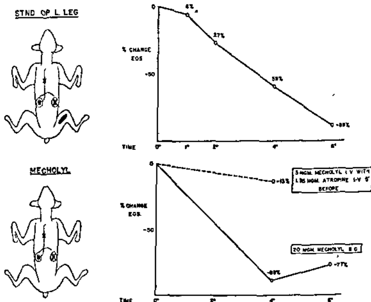


FIG 7. The eosinopenic response to operative trauma and [illegible]

atropine

reaching the general circulation, and a less marked eosinopenia should develop. Actually a given dose of Compound F produced the same degree of eosinopenia in the adrenalectomized dog with or without trauma. Under the conditions of this experiment, therefore, it appeared that the traumatized

by a direct action of the corticoids on the anterior pituitary, as postulated by Sayers (1947). In the dog, for example, injecting large amounts of deoxycorticosterone glucoside intravenously will prevent the release of ACTH following trauma, or even hypothalamic stimulation. The rôle of the hypothalamus seems to be to initiate an increased secretion of ACTH in response to stresses of various kinds, and this increased secretion is probably brought under control ultimately by increased blood level of corticoids. Hypothalamic stimulation is brought about by a variety of different stresses and therefore serves as a co-ordinating mechanism between non-specific stresses and the specific release of pituitary ACTH. The effect of the hypothalamus on the pituitary appears to be mediated by a humoral substance. Hypothalamic stimulation also results in the release of epinephrine from the adrenal medulla and this, in addition to its other functions, appears able directly to stimulate the hypothalamus. It is realized that there are probably other factors which are important in the release of ACTH and this diagram is vastly over-simplified. Much work remains to be done to elucidate further the rôle of the hypothalamus in the release of anterior pituitary hormones, but we feel that the hypothalamus has been shown to be an important factor in the release of ACTH from the anterior pituitary.

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Summary and Conclusions

Fig. 9 outlines our present concept about one means by which ACTH is released from the pituitary following stress. This theory may be modified considerably by the results of

FIG. 9 A diagram illustrating concept of pathways involved in the increased secretion of ACTH following stress Peripheral nervous impulses, humoral substances (epinephrine, insulin, ? tissue product) and cortical impulses are represented as ending in hypothalamic stimulation, which in turn leads to the release of a humoral substance that acts on the pituitary to release ACTH. [illegible] by the [illegible] promotes.

further experimentation and it represents merely a working hypophysis to try to explain the findings which we have observed in our experiments to date. There is a balance between the pituitary secretion of ACTH and the adrenal cortical secretion of corticoids which is probably maintained

HUME: We have done some work with Nembutal in this regard, which I didn't want to bring in now because it's a rather lengthy discussion. The effects of Nembutal seem to be quite variable. The eosinopenia following epinephrine and operative trauma are sometimes decreased by Nembutal, and sometimes not. Nembutal anæsthesia alone produces a slight eosinopenia in some animals and not in others, etc.

HARRIS: In regard to the speed with which ACTH is secreted in response to a stimulus, there was a recent paper by Gray and Munson (Gray, W. D., and Munson, P. L., 1951. *Endocrinology*, 48, 471) in which they injected histamine in rats and found that the release of ACTH was stimulated within 10 seconds of the injection.

HUME: Yes, that's right. The shortest time we used was a minute, and it's certainly true that it's under a minute.

HARRIS: A minute would be too short for Sayers's suggested mechanism to work.

LONG: But you still have to account for the fact that, as Gordon has shown in rats, even in the deafferentated limb severe trauma will still produce a fall in the adrenal ascorbic acid.

HUME: That's got to be a sympathetically denervated limb also, because if you simply denervate the somatic afferents, impulses can apparently travel up the sympathetic afferents.

LONG: But you still get a fall.

HUME: With severe trauma you still get a minimal fall. We get a fall with burn trauma, which is a very severe trauma, and, as I said, there must have been some spinal release of epinephrine in response to this burn trauma.*

LONG: It seems to me we still have epinephrine coming into this picture—sympathetic stimulation is there. You are postulating there is a type of reflex stimulation of the hypothalamic area that can only involve the anterior portion. In these circumstances, you *do* get epinephrine secretion as well.

HUME: You do, that's right, but ACTH is released in response to trauma whether epinephrine is present or not, as we have shown by sympathectomized adrenal-demedullectomized animals.

BROBECK: Certain points were not quite clear to me. One of them had to do with your second slide. The first animal there, with a lesion that was entirely in the posterior hypothalamus, was the one that showed the least response in the eosinophils.

HUME: That difference in response is an apparent and not a real difference. When the responses are around +34 per cent instead of around +10 per cent it doesn't mean that the animal is less responsive.

We thought that perhaps we were destroying afferent tracts leading

*Later experiments have shown that this minimal eosinopenia follows burn trauma even in the *sympathectomized, denervated limb—perhaps by virtue* of something in the tissue released by a severe burn.

DISCUSSION

LONG: Dr Hume, you have relied entirely here on the eosinophil [illegible] as a measurement of the release of ACTH. Have you had the

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we have come to

fter all, there is a

adrenal cortical

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influence the eosinophil test

method that is

that if the animal has been previously operated on you get rather bizarre behaviour of the eosinophils. In fact, you can't say that you always get a fall in the eosinophils in an animal that has had a previous trauma. It's true also of the adrenal ascorbic acid. It can at times be completely deceiving.

HUME. I think that most of those differences will level themselves out, because we kept all of these animals for at least four months and we made several tests on every animal.

LONG: Does adrenal atrophy occur?

HUME: No. That's one of the differences between this animal and

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demonstrated in various ways is not always the same. For instance, if you accept the fact that Nembutal depresses the hypothalamus you will find that all functions are not equally depressed For instance,

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HYPOTHALAMIC CONTROL OF ACTH SECRETION BY THE PITUITARY GLAND

J. DE GROOT and G. W. HARRIS

There is much evidence that the activity of most of the endocrine glands may be affected by changes in the external environment. It is well known that the functional activity of the ovaries and testes may be modified by changes in light exposure, that the activity of the thyroid gland is influenced by the environmental temperature, and that various forms of stress exert a marked effect on the functions of the adrenal cortex. It is very probable that these effects are mediated by the anterior pituitary gland.

In 1947 we started investigating the mechanism by which the secretion of ACTH from the anterior pituitary gland is controlled. At this time it was known that increased secretion of ACTH could be gauged either by noting the direct effects of the hormone on the adrenal cortex (structure and weight of the gland, cholesterol and ascorbic acid content of the cortex) or by observing the indirect effects which follow increased adrenal cortical secretion (transient lymphopenia).

Preliminary experiments, in collaboration with Dr. Harry Colfer, showed that normal rabbits react to emotional stress (immobilization or subcutaneous faradism) with a reduction in the number of circulating lymphocytes (Colfer, de Groot and Harris, 1950). This lymphopenia was maximal in about three hours and could be closely simulated in magnitude and time relations by an injection of ACTH. Hypophysectomized rabbits exposed to the same stresses did not show any response, though injection of ACTH was still followed by a lymphopenia. Denervation of the adrenal glands did not modify the response. There was then available for investigation in the rabbit a reaction which could be expressed:

only the supraoptic nuclei we have not had any decrease in the response to stress at all—it's been perfectly normal—and it's only the lesions which have involved the wall of the third ventricle and the floor, and one or two animals in which the posterior portion of the floor of the third ventricle was destroyed, which have had a decrease in their response to stress.

BROBECK: A chart of this type suggests to some of us the interruption of an efferent pathway leaving the hypothalamus through the caudal part rather than of an afferent path coming to it

What is the evidence that these high spinal sections are accomplishing their effect by sympathetic rather than by afferent denervation? Have you done any purely sympathetic denervation along with the lower spinal lesions?

HUME: *I have begun the operations, but have no results to report yet.* It is interesting that you apparently have to keep going up the cord until you get to the point at which you're just above the place where the last sympathetic enters the cord before you get the absence of response

BROBECK We have had some experience with dogs recently, and it's our impression that although this mechanism may be active in a very few minutes, as you described from your blocking experiments, nevertheless the eosinophil response takes a long time, and in some cases hours. *Is it possible that by using the dog, where the eosinophils are* either more stable or their destruction is somehow slower, that one may be missing several parts of a mechanism that may be responding more quickly?

HUME: I don't think so, because if you give intravenous ACTH either in the dog or the human the time and the shape of the curve is about the same as if you applied a stimulus to either the dog or the human.

LONG: But it's slow in the dog. It takes four hours with ACTH.

HUME: Yes, it's slow with both of them It's slow even with intravenous ACTH. That means that since intravenous ACTH gives the same curve as a stimulus does, that the lag can't be between the stimulus and the release of ACTH.

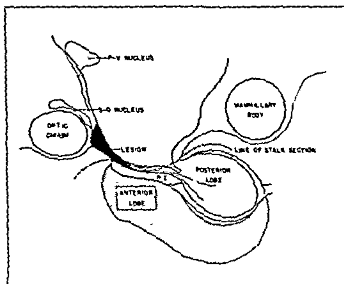
LONG: Have you any experience with histamine in the dog? Because it was shown in rats that the response of eosinophils to histamine was as great in 15 minutes as the response to intravenous ACTH was in four hours

HUME: We've done very little with histamine in the dog In the experiments in the rat the doses of histamine which have been used were so outside the physiological limits as to raise grave doubts as to their validity. *In the dog we've used only up to 2 ml. of 1·1,000 histamine* intravenously, which gives the dog a marked systemic reaction. This dose produces no eosinophil fall in dogs.

to stress. We have as yet no explanation for the observation that the animals begin to respond again after a long period of time.

The pre- and post-operative response of a typical animal with a hypothalamic lesion is shown in Fig. 2.

Section of the stalk along the plane shown by the dotted line in diagram 1 did not prevent the normal eosinopenic



response following stress. Serial sections of the hypothalamus and the pituitary were made in these animals but indian ink injections of the blood vessels were not done. It cannot, therefore, be conclusively stated that the hypophyseal portal vessels had not regrown. However, one of the animals was tested only a week after the operation, and a polyeth

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emotional stress → stimulation of the central nervous system → excitation of the anterior pituitary → excitation of the adrenal cortex → lymphopenia.

It was decided to investigate the pathway by which the stimulus from the central nervous system is transmitted to the anterior pituitary gland. For this purpose, electrolytic lesions were placed in various parts of the hypothalamus and pituitary gland and the effect of such lesions on the lymphopenic response to stress was studied (de Groot and Harris, 1950). It was found that lesions in the anterior pole of the pituitary (zona tuberalis) abolished the response, and that transverse lesions in the posterior part of the tuber cinereum and mammillary body diminished or abolished the response. Similar lesions elsewhere in the pituitary gland or hypothalamus were compatible with normal lymphopenic responses.

The converse experiments (de Groot and Harris, 1950) consisted of electrical stimulation of various parts of the hypothalamus and the pituitary gland, to see whether this resulted in a lymphopenia comparable to that evoked by stress or injection of ACTH. For this purpose a technique of electrical stimulation was required which did not entail any *incidental emotional stress during its application.* The remote control method of stimulation was therefore used. In a preliminary operation a small coil was implanted between the skull and the scalp and an insulated electrode led from one end of the coil into the required region. The tissue surrounding the bare electrode tip could then be stimulated by passing *high amperage current pulses through a large primary coil* surrounding the rabbit's cage. The buried secondary coil was thereby situated in an electromagnetic field, the strength of which could be easily adjusted. Thus a localized electrical stimulus could be applied in the hypothalamo-hypophyseal region in unanæsthetized, unrestrained rabbits. Using this technique it was *found* that stimulation of the posterior tuber cinereum or mammillary body resulted in a lymphopenia similar to that following emotional stress or intravenous injections of an appropriate dose of ACTH. Stimulation of

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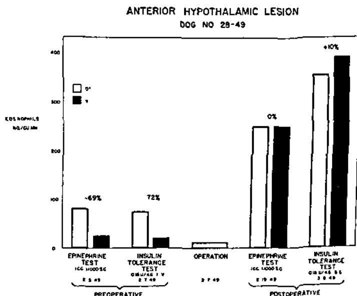


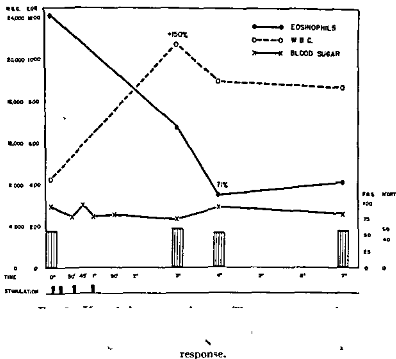
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DOG NO 173-COIL IMPLANT



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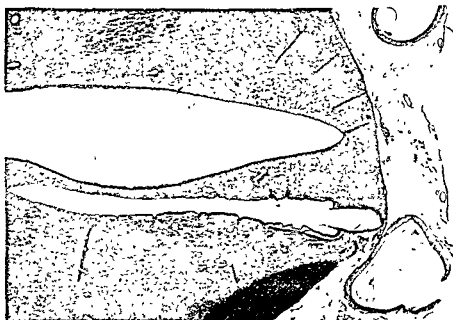


Fig 4 Photomicrograph of coronal section through hypothalamus in dog No 173-49 showing the location of the tip of the stimulating electrode The bare tip of the platinum wire

seen that the marked eosinopenia and rise in white count following hypothalamic stimulation occur even without release of endogenous epinephrine.

To summarize the foregoing experiments: It was possible to make lesions in the hypothalamus which abolished the release of ACTH from the pituitary following stress although the pituitary itself was intact. Conversely, stimulation of the hypothalamus produced a release of ACTH from the pituitary even in the absence of the sympathetics. Section of the stalk did not appear to prevent the release of ACTH following stress. The development or absence of diabetes insipidus had no effect on ACTH release. We concluded from these experiments: (1) That the hypothalamus was important in the increased release of ACTH from the pituitary which occurs following stress; (2) That its effect on the pituitary need not be brought about by epinephrine or by pituitrin; and (3) That its effect must be mediated by some humoral substance because it was present even in the absence of any direct connection between the pituitary and the hypothalamus.

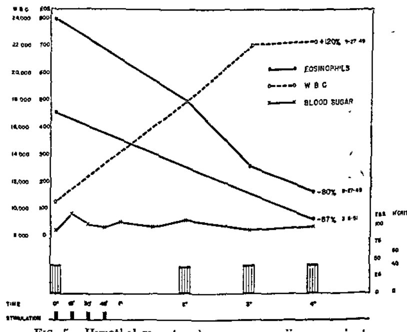
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The Effects of Section of the Spinal Cord on the Release of Pituitary ACTH Following Trauma to the Denervated Area

This is a difficult group of animals to work with and the results are not always consistent. There is some evidence that sympathetic, as well as somatic, afferents have to be eliminated, so that the limb is completely denervated, before one notes an absence of ACTH release following trauma to the denervated extremity. Fig. 6 shows the results of some

It is known that stimulation of the posterior hypothalamus leads to sympathetic excitation and thus to release of epinephrine from the adrenal medulla. Although we have not observed any evidences of epinephrine release following



stimulation periods done 18 months apart are shown There is no appreciable change in hæmatocrit or blood sugar.

stimulation of the anterior hypothalamus, we had not completely ruled out epinephrine as the cause of the eosinopenia observed after hypothalamic stimulation. Therefore, coils were implanted in totally sympathectomized dogs. A diagram of the effects of hypothalamic stimulation in a totally sympathectomized dog is shown in Fig. 5. It may be

fall which was noted. A study is under way on sympathectomized dogs to check this point.*

Other Possible Mechanisms of ACTH Release

It is well known that epinephrine can produce a release of ACTH from the pituitary, and from the results of our experimentation it appears to us that this must be due to the effect of epinephrine on the hypothalamus rather than by a direct action on the pituitary, as postulated by McDermott *et al.* (1950). It has been shown above that the release of ACTH following hypothalamic stimulation does not depend on the presence of epinephrine. It was likewise shown, as may be seen in Fig. 7, by using a sympathectomized, unilaterally adrenalectomized, unilaterally adrenal-demedullectomized dog, that operative trauma produced a marked ACTH release even in the absence of epinephrine, as shown by the eosinopenia following this procedure. An injection of mecholyl likewise produced a marked eosinopenia in the absence of epinephrine. This response was abolished by injecting atrophine before injecting mecholyl. It has also been shown that the injection of insulin produces a marked eosinopenia in the absence of epinephrine.

An experiment was done to check the theory of Sayers (1947) that the traumatized area removes corticoids from the blood stream, thereby decreasing the amount of circulating corticoids and inducing ACTH release. A bilaterally adrenalectomized dog maintained on a DCA pellet was used for these experiments. An injection of Compound F was given intra-arterially and a dose was determined that was small enough to give just a significant fall of eosinophils (Fig. 8). The leg of this dog was now traumatized at operation, a procedure which of course produces no eosinopenia in an adrenalectomized dog, and the same amount of Compound F was now

*Subsequent experiments have shown that a sub-normal eosinopenic response can be produced by a severe burn below the level of cord section even after total sympathectomy. This may be due to direct action of the products of tissue destruction upon the adrenal medulla or upon the hypothalamus.

experiments on a dog with a cord section at the level of C7, and it will be noted that he no longer showed an eosinopenia following operative trauma to the denervated extremity, although he continued to show an eosinopenia following injections of epinephrine and to operative trauma above the

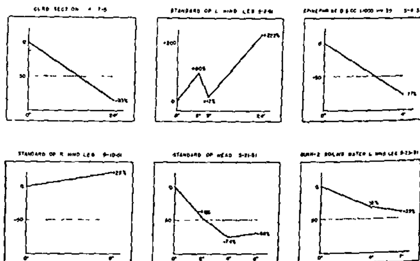


FIG 6. The percentage change in eosinophils in response to trauma below and above the level of cord section. The response to I-V epinephrine is also charted No eosinopenia was induced by operative trauma to the denervated portion of the body, although a similar procedure done to the head produced a normal eosinopenic response Severe trauma (burn) to the leg produced a minimal eosinopenic response, probably on the basis of reflex secretion of endogenous epinephrine

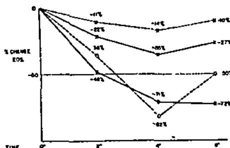
level of cord section. Very severe burn trauma to the hind leg did produce a slight eosinopenia, though far less than one would see following such trauma in an intact animal. This animal was not sympathectomized and he developed marked mass reflexes when his leg was put in boiling water. This may have released enough endogenous epinephrine to produce the

area did not directly take Compound F from the blood stream. These experiments were carried out on a single adrenalectomized dog, and more animals will have to be studied to establish this point finally. The experiments cited above in

DOG NO 33-51 BILATERAL ADRENALECTOMY + DCA PELLET

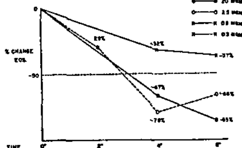
COMPOUND F I-A





ETHER + TRAUMA + CPD F I-A





which moderate trauma to the denervated extremity failed to produce a significant eosinopenia likewise suggest that direct utilization of corticoids by injured tissues was not the principal factor responsible for ACTH release under these circumstances.

injected intra-arterially so that it would come in contact with the traumatized area before gaining access to the general circulation. If the Compound F were removed from the blood stream by the traumatized area there should be less of it

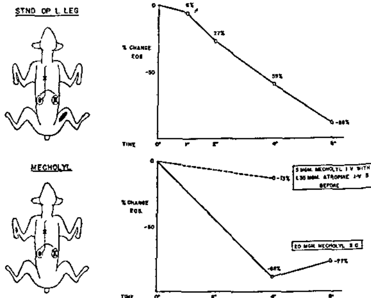


FIG. 7. The eosinopenic response to operative trauma and

atropine

reaching the general circulation, and a less marked eosinopenia should develop. Actually a given dose of Compound F produced the same degree of eosinopenia in the adrenalectomized dog with or without trauma. Under the conditions of this experiment, therefore, it appeared that the traumatized

by a direct action of the corticoids on the anterior pituitary, as postulated by Sayers (1947). In the dog, for example, injecting large amounts of deoxycorticosterone glucoside intravenously will prevent the release of ACTH following trauma, or even hypothalamic stimulation. The rôle of the hypothalamus seems to be to initiate an increased secretion of ACTH in response to stresses of various kinds, and this increased secretion is probably brought under control ultimately by increased blood level of corticoids. Hypothalamic stimulation is brought about by a variety of different stresses and therefore serves as a co-ordinating mechanism between non-specific stresses and the specific release of pituitary ACTH. The effect of the hypothalamus on the pituitary appears to be mediated by a humoral substance. Hypothalamic stimulation also results in the release of epinephrine from the adrenal medulla and this, in addition to its other functions, appears able directly to stimulate the hypothalamus. It is realized that there are probably other factors which are important in the release of ACTH and this diagram is vastly over-simplified. Much work remains to be done to elucidate further the rôle of the hypothalamus in the release of anterior pituitary hormones, but we feel that the hypothalamus has been shown to be an important factor in the release of ACTH from the anterior pituitary.

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Summary and Conclusions

Fig. 9 outlines our present concept about one means by which ACTH is released from the pituitary following stress. This theory may be modified considerably by the results of

FIG. 9 A diagram illustrating concept of pathways involved in the increased secretion of ACTH following stress. Peripheral nervous impulses, humoral substances (epinephrine, insulin,

further experimentation and it represents merely a working hypophysis to try to explain the findings which we have observed in our experiments to date. There is a balance between the pituitary secretion of ACTH and the adrenal cortical secretion of corticoids which is probably maintained

HUME: We have done some work with Nembutal in this regard, which I didn't want to bring in now because it's a rather lengthy discussion. The effects of Nembutal seem to be quite variable. The eosinopenia following epinephrine and operative trauma are sometimes decreased by Nembutal, and sometimes not. Nembutal anæsthesia alone produces a slight eosinopenia in some animals and not in others, etc.

HARRIS: In regard to the speed with which ACTH is secreted in response to a stimulus, there was a recent paper by Gray and Munson (Gray, W. D., and Munson, P. L., 1951. *Endocrinology*, 48, 471) in which they injected histamine in rats and found that the release of ACTH was stimulated within 10 seconds of the injection.

HUME: Yes, that's right. The shortest time we used was a minute, and it's certainly true that it's under a minute.

HARRIS: A minute would be too short for Sayers's suggested mechanism to work.

LONG: But you still have to account for the fact that, as Gordon has [illegible] auma will still [illegible] ted limb also, [illegible] impulses can [illegible]

[illegible] must have been some spinal release of epinephrine in response to this burn trauma.*

LONG: It seems to me we still have epinephrine coming into this picture—sympathetic stimulation is there. You are postulating there is a type of reflex stimulation of the hypothalamic area that can *only* involve the anterior portion. In these circumstances, you *do* get epinephrine secretion as well.

HUME: You do, that's right, but ACTH is released in response to trauma whether epinephrine is present or not, as we have shown by sympathectomized adrenal-demedullectomized animals.

BROBECK: Certain points were not quite clear to me. One of them had to do with your second slide. The first animal there, with a lesion that was entirely in the posterior hypothalamus, was the one that showed the least response in the eosinophils.

HUME: That difference in response is an apparent and not a real difference. When the responses are around +34 per cent instead of around +10 per cent it doesn't mean that the animal is less responsive. [illegible]

*Later experiments have shown that this minimal eosinopenia follows burn trauma even in the sympathectomized, denervated limb—perhaps by virtue of something in the tissue released by a severe burn.

DISCUSSION

LONG: Dr. Hume, you have relied entirely here on the eosinophil

very good direct method for the determination of adrenal cortical steroids, and we know comparatively little about the factors that influence the eosinophil test.

HUME: That's very true. There are objections to any method that is

LONG It's true under certain circumstances, but it is also a fact that if the animal has been previously operated on you get rather bizarre behaviour of the eosinophils. In fact, you can't say that you always get a fall in the eosinophils in an animal that has had a previous trauma. It's true also of the adrenal ascorbic acid. It can at times be completely deceiving.

HUME: I think that most of those differences will level themselves out, because we kept all of these animals for at least four months and we made several tests on every animal

LONG: Does adrenal atrophy occur?

HUME: No. That's one of the differences between this animal and

with an increase in sensitivity

LONG: You postulated an effect of epinephrine solely upon the hypothalamic centres. I presume you regard epinephrine as a stimulus to the hypothalamic centres Well, has that ever been shown? It was my impression that epinephrine does *not* act as a stimulus to the hypothalamic centres. In fact I think there is some evidence that it actually decreases the response of the hypothalamic centres

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functions. We think that epinephrine works on the hypothalamus

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HYPOTHALAMIC CONTROL OF ACTH SECRETION BY THE PITUITARY GLAND

J. DE GROOT and G. W. HARRIS

THERE is much evidence that the activity of most of the endocrine glands may be affected by changes in the external environment. It is well known that the functional activity of the ovaries and testes may be modified by changes in light exposure, that the activity of the thyroid gland is influenced by the environmental temperature, and that various forms of stress exert a marked effect on the functions of the adrenal cortex. It is very probable that these effects are mediated by the anterior pituitary gland.

In 1947 we started investigating the mechanism by which the secretion of ACTH from the anterior pituitary gland is controlled. At this time it was known that increased secretion of ACTH could be gauged either by noting the direct effects of the hormone on the adrenal cortex (structure and weight of the gland, cholesterol and ascorbic acid content of the cortex) or by observing the indirect effects which follow increased adrenal cortical secretion (transient lymphopenia).

Preliminary experiments, in collaboration with Dr. Harry Colfer, showed that normal rabbits react to emotional stress (immobilization or subcutaneous faradism) with a reduction in the number of circulating lymphocytes (Colfer, de Groot and Harris, 1950). This lymphopenia was maximal in about three hours and could be closely simulated in magnitude and time relations by an injection of ACTH. Hypophysectomized rabbits exposed to the same stresses did not show any response, though injection of ACTH was still followed by a lymphopenia. Denervation of the adrenal glands did not modify the response. There was then available for investigation in the rabbit a reaction which could be expressed:

only the supraoptic nuclei we have not had any decrease in the response to stress at all—it's been perfectly normal—and it's only the lesions which have involved the wall of the third ventricle and the floor, and one or two animals in which the posterior portion of the floor of the third ventricle was destroyed, which have had a decrease in their response to stress.

BROBECK: A chart of this type suggests to some of us the interruption of an efferent pathway leaving the hypothalamus through the caudal part rather than of an afferent path coming to it.

What is the evidence that these high spinal sections are accomplishing their effect by sympathetic rather than by afferent denervation? Have you done any purely sympathetic denervation along with the lower spinal lesions?

HUME. I have begun the operations, but have no results to report yet. It is interesting that you apparently have to keep going up the cord until you get to the point at which you're just above the place where the last sympathetic enters the cord before you get the absence of response.

BROBECK: We have had some experience with dogs recently, and it's our impression that although this mechanism may be active in a very few minutes, as you described from your blocking experiments, nevertheless the eosinophil response takes a long time, and in some cases hours. Is it possible that by using the dog, where the eosinophils are either more stable or their destruction is somehow slower, that one may be missing several parts of a mechanism that may be responding more quickly?

HUME: I don't think so, because if you give intravenous ACTH either in the dog or the human the time and the shape of the curve is about the same as if you applied a stimulus to either the dog or the human.

LONG. But it's slow in the dog. It takes four hours with ACTH.

HUME. Yes, it's slow with both of them. It's slow even with intravenous ACTH. That means that since intravenous ACTH gives the same curve as a stimulus does, that the lag can't be between the stimulus and the release of ACTH.

LONG. Have you any experience with histamine in the dog? Because it was shown in rats that the response of eosinophils to histamine was as great in 15 minutes as the response to intravenous ACTH was in four hours.

HUME. We've done very little with histamine in the dog. In the experiments in the rat the doses of histamine which have been used were so outside the physiological limits as to raise grave doubts as to their validity. In the dog we've used only up to 2 ml of 1:1,000 histamine intravenously, which gives the dog a marked systemic reaction. This dose produces no eosinophil fall in dogs.

other parts of the hypothalamus (including the supraoptico-hypophyseal tract) or of any region of the pituitary gland was not followed by a lymphopenia.

From this and other evidence it was concluded that the pathway by which stimuli from the central nervous system excite increased ACTH secretion from the pituitary gland consists of a neural and a vascular component The former component, which is situated in the posterior part of the tuber cinereum and mammillary region, may be blocked by lesions and excited by localized electrical stimulation. The latter or vascular component, the hypophyseal portal vessels, extends from the median eminence of the tuber cinereum through the zona tuberalis to the pars distalis. It is reasonable to suppose that these structures may be blocked by lesions but are inexcitable to electrical stimuli. These conclusions are also supported by the findings of anatomical and histological studies of this region in normal animals.

The *secretion of ACTH by the pituitary gland seems to be* under neural control via the posterior hypothalamus and the hypophyseal portal vessels of the pituitary stalk.

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emotional stress → stimulation of the central nervous system → excitation of the anterior pituitary → excitation of the adrenal cortex → lymphopenia.

It was decided to investigate the pathway by which the stimulus from the central nervous system is transmitted to the anterior pituitary gland. For this purpose, electrolytic lesions were placed in various parts of the hypothalamus and pituitary gland and the effect of such lesions on the lymphopenic response to stress was studied (de Groot and Harris, 1950). It was found that lesions in the anterior pole of the pituitary (zona tuberalis) abolished the response, and that transverse lesions in the posterior part of the tuber cinereum and mammillary body diminished or abolished the response. Similar lesions elsewhere in the pituitary gland or hypothalamus were compatible with normal lymphopenic responses.

The converse experiments (de Groot and Harris, 1950) consisted of electrical stimulation of various parts of the hypothalamus and the pituitary gland, to see whether this resulted in a lymphopenia comparable to that evoked by stress or injection of ACTH. For this purpose a technique of electrical stimulation was required which did not entail any incidental emotional stress during its application. The remote control method of stimulation was therefore used. In a preliminary operation a small coil was implanted between the skull and the scalp and an insulated electrode led from one end of the coil into the required region. The tissue surrounding the bare electrode tip could then be stimulated by passing high amperage current pulses through a large primary coil surrounding the rabbit's cage. The buried secondary coil was thereby situated in an electromagnetic field, the strength of which could be easily adjusted. Thus a localized electrical stimulus could be applied in the hypothalamo-hypophyseal region in unanæsthetized, unrestrained rabbits. Using this technique it was found that stimulation of the posterior tuber cinereum or mammillary body resulted in a lymphopenia similar to that following emotional stress or intravenous injections of an appropriate dose of ACTH. Stimulation of

pathway laid down in the pars tuberalis, and it has been suggested that this part of the pituitary, which has no proven endocrine function, is established in the embryo to form the bed for such a pathway (Harris, 1947*a*). On the basis of what is known regarding transmission of stimuli in general it might be expected that the hypothalamus stimulates the anterior pituitary gland by (*a*) nerve fibres, or (*b*) humoral channels, laid down in this path.

1. Innervation of the Anterior Pituitary Gland

Most workers have found that the pars distalis of the adenohypophysis receives a very scanty innervation, if any at all. In view of the danger of mistaking reticular connective tissue fibres for nerve fibres, any claim that the gland receives a rich innervation requires the simple control procedure of staining sections from a "denervated" gland on the same slide as sections from a normal gland. At the moment there is no sound evidence for believing that the pars distalis of the pituitary receives secretomotor nerve fibres (Harris, 1948*a*).

2. Vascular Supply of the Anterior Pituitary Gland

The adenohypophysis has a systemic arterial supply in the form of small arterial twigs from the internal carotid artery or from the Circle of Willis, and systemic veins which pass into the surrounding venous sinuses.

Many years ago Professor Fr. I. Rainer of Bucharest noticed that human subjects, at autopsy, sometimes showed a prominent system of blood-vessels running along the pituitary stalk (see Popa and Fielding, 1935). It was found that these vessels are markedly engorged with blood immediately after death, and especially so when the death was of a violent nature. The same observation was made afterwards on animals killed at the slaughter house. One of Rainer's pupils (afterwards Professor Popa) made a microscopic study of these vessels and found that they were portal vessels, in that the large trunks of the pituitary stalk passed into capillaries if traced upwards to the hypothalamus or downwards into the

HYPOTHALAMIC CONTROL OF THE ANTERIOR PITUITARY GLAND

G. W. HARRIS

THE factors which maintain and control the rate of secretion of hormones from the anterior pituitary gland have been the subject of many investigations. These factors occupy a position of some importance in the field of endocrinology since they determine also the activity of the target endocrines (ovaries, testes, thyroid and adrenal cortex) upon which the anterior pituitary hormones exert their action.

It may be significant, in this respect, that Rathke's pouch in the embryo becomes detached from the epithelium of the stomodæum and migrates, through what will later become the base of the skull, to attain a final resting place in the cranium. From the teleological point of view, it might be suggested that the purpose of this embryonic migration of the adenohypophysis to the base of the brain is the formation of an anatomical link between the two structures, by means of which the central nervous system of the adult regulates anterior pituitary activity. The most constant point of contact between the derivatives of Rathke's pouch on the one hand, and the derivatives of the hypothalamus on the other, lies in the contact of the pars tuberalis with the median eminence of the tuber cinereum. This has been observed in nearly all vertebrates examined and is brought about by two small evaginations of Rathke's pouch, the lateral lobes, which ascend and in most forms encircle the tuber cinereum to form the pars and zona tuberalis of the gland. In some forms, such as the *Cetacea*, this is the only point of anatomical union between the central nervous system and the adenohypophysis. It would seem likely then that any influence the nervous system exerts over this gland would be mediated by a

Taubenhaus and Soskin (1941), on the basis of experiments in which they applied a prostigmine-acetylcholine mixture to the exposed pituitary glands of rats, carried the idea a step further and suggested the humoral stimulus to the anterior pituitary might be carried by the hypophyseal portal vessels. This possibility was considered in more detail, and the evidence regarding it summarized by Harris (1944), and after a further study of the vessels the hypothesis was restated by Green and Harris (1947). At the present time it can be said that these vessels are intimately related to the maintenance and control of anterior pituitary activity. The experimental evidence for this statement will now be considered.

(*a*) *Electrical stimulation of the hypothalamus elicits anterior pituitary secretion whereas stimulation of the gland itself does not.* It is well known that in the rabbit the stimulus of coitus excites the adenohypophysis, by means of a nervous reflex, to secrete gonadotrophic (luteinizing) hormone and that ovulation thereby occurs some ten hours after mating. On the supposition that one part of the path of the nervous reflex involved the hypothalamus, this structure and the pituitary gland have been subjected to electrical stimulation. Markee, Sawyer and Hollinshead (1946) found that ovulation is more easily elicited by electrical stimulation of the hypothalamus than of the hypophysis. At the time of this report the same result had been obtained (Harris, 1948*b*) in experiments in which localized electrical stimuli had been applied to the same structures in a number of unanæsthetized rabbits. The method used was that of remote control stimulation (Harris, 1947*b*), and the stimulus applied had a spread of not more than ½ mm. so far as unmyelinated fibres were concerned. Stimulation of the various parts of the hypothalamus and hypophysis was performed in seventeen rabbits for periods varying from 1 minute to 7½ hours. Up to three experiments were performed on each animal. In the first experiment the period of stimulation chosen was usually 1–3 hours, followed by laparotomy 48 hours later. After a month the experiment was repeated with an increased or decreased

pituitary gland. This work was interrupted and not published. Some years later, at the suggestion of Professor G. Elliot-Smith, the anatomy of these vessels was reinvestigated at University College, London. Popa and Fielding (1930, 1933) confirmed that these vessels were really portal in nature, and suggested that blood was drained from both lobes of the pituitary gland up the stalk and redistributed in a "secondary net" of capillaries in the hypothalamus. The presence of these vessels in other forms was reported by Wislocki and King (1936) and Wislocki (1937, 1938). These workers differed from Popa and Fielding in believing that the direction of blood flow was towards the pituitary gland, that the upper net or plexus of capillaries was situated in the median eminence of the tuber cinereum (that is in tissue of the neurohypophysis rather than hypothalamus proper), and that the lower end of the vessels connected with the sinusoids of the anterior lobe only of the pituitary. Green and Harris (1947) found similar portal vessels in the common laboratory animals—dogs, rabbits, guinea-pigs and rats—as well as in man. Direct microscopic examination of the vessels in living amphibians (Houssay, Biasotti and Sammartino, 1935; Green, 1947), and rats (Green and Harris, 1949) has established that the blood flows *from* the primary plexus of capillaries in the median eminence, through the trunks of the vessels situated largely in the pars *tuberalis of the stalk, to the sinusoids of the pars distalis* In the last few years a detailed study has been made of the hypophyseal portal system in a wide variety of vertebrates (Green, 1951).

3. Evidence Relating the Hypophyseal Portal Vessels to the Control of Anterior Pituitary Activity

Over ten years ago Hinsey and Markee (1935), Harris (1937) and Brooks (1938) mentioned, rather tentatively, the possibility that a stimulus might be transmitted from the hypothalamus or neurohypophysis to the adenohypophysis "humorally." This idea was put forward in view of the paucity of identifiable nerve fibres in the anterior pituitary.

fifty-five rats. After operation their reproductive functions were studied. In twenty-three of these animals the stalk was divided and the ends left in proximity; of these, fourteen showed the return of a regular œstrous rhythm, eight the return of an irregular rhythm and one remained permanently anœstrous. Thirty-two rats had the stalk divided and a foreign body (usually a small paper plate) inserted between the cut ends of the stalk, and of these fifteen remained permanently anœstrous. The majority of animals that showed a post-operative return of œstrous cycles were tested with a sterile buck, and showed a pseudo-pregnancy response (and decidual reaction) following sterile coitus. At the end of the experiment the animals were killed, the vascular tree perfused with indian ink and serial sections cut through a block of tissue containing the pituitary gland and hypothalamus. A study of these sections revealed: firstly, that the hypophyseal portal vessels regenerate across the site of stalk section with what appears to be extreme facility; and secondly, that there was a correlation between the return of reproductive functions after operation and the amount of regeneration of these vessels. For example, it was found in seventeen rats (in which a foreign body had been inserted between the cut ends of the stalk and which had returned to œstrus after operation) that regeneration of the portal vessels had occurred through the interstices of wool plugs, and around the borders of the wax or paper plates when these had been slightly misplaced. The injection of the vessels with indian ink, and the sectioning of a block of tissue with any plate still *in situ*, would seem to be essential control procedures before any statement can be made regarding the complete and permanent interruption of these vessels after pituitary stalk section. In a further group of eighteen rats the stalk was sectioned and a study made of the rate of regeneration of the portal vessels. Regeneration was observed to start within one day of operation. The hypophyseal portal vessels of the monkey have been observed to regenerate in a similar manner to those of the rat (Harris and Johnson, 1950).

duration of stimulus according to the previous result. In most cases a third experiment was performed, the animal then being killed. It was found that out of seventeen rabbits, eight gave the ovulatory response to stimulation and nine were negative. In the eight positive cases the electrode was situated in some part of the tuber cinereum and in the nine negative in some part of the adenohypophysis. The contrast between the excitability of the hypothalamus with that of the pituitary gland may be illustrated by the fact that one rabbit that had the electrode situated in the anterior part of the tuber cinereum gave a full ovulatory response to 3 minute stimulation, whereas other rabbits in which the electrode was situated in various parts of the adeno- or neuro-hypophysis gave no sign of ovarian activation after 7½ hours stimulation. The conclusion drawn from these results is that coitus in the rabbit normally excites the release of gonadotrophins by stimuli which reach the region of the tuber cinereum by nervous pathways. From the tuber cinereum to the anterior lobe of the pituitary the path is inexcitable to electrical stimuli, and the stimulus is probably transmitted humorally in this region.

Similar results and conclusions were reached after a study of the effects of such stimuli in causing the release of a glycotrophic factor and of the adrenocorticotrophic hormone (see Harris, 1948*a*, and de Groot and Harris, 1950).

(*b*) *The variable effects of section of the hypophyseal stalk on anterior pituitary function.*

Results obtained by different workers from experiments involving section of the pituitary stalk show marked discrepancies. Very varied conclusions have been drawn as to the importance of an intact pituitary stalk in relation to the functions of the anterior pituitary gland. It was thought that these discrepancies might be explained by different degrees of regeneration of the hypophyseal portal vessels following the initial stalk section. To test this idea, a temporal approach to the pituitary region of the rat was developed (Harris, 1950) and the pituitary stalk divided in a group of

4. Conclusions Regarding Hypothalamic Control of Anterior Pituitary Function

The conclusions drawn are represented diagrammatically in Fig. 1. The hypothalamus is regarded as a region where the effect of neural stimuli, arising in the external environment and in the higher centres of the brain, are integrated with

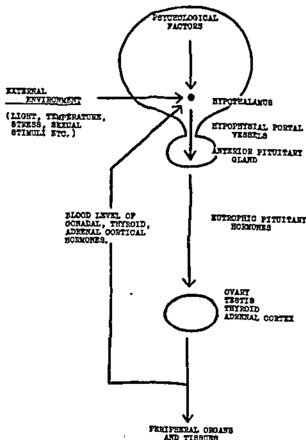


FIG. 1. Diagram to illustrate [illegible]

(*c*) *Evidence derived from a study of anterior pituitary transplants and grafts.*

Most endocrine glands, including the ovary, testis, thyroid and adrenal cortex, may be transplanted to a distant site in the body and maintain a high degree of functional activity. It seems likely that this is due to the fact that they derive their normal stimulus to activity, the pituitary eutrophic hormones, through the general systemic circulation, and this reaches them effectively after transplantation. Many studies have been made on the functional activity of anterior pituitary transplants, and the position was summarized by Ingle and Griffith (1942) when they stated—"Viable grafts of anterior lobe tissue may be obtained but no method has been established whereby such grafts can be made to maintain a normal level of functional activity." On the theory that the anterior pituitary gland receives its stimulus to normal activity through the hypophyseal portal vessels, it would seem reasonable to suppose that transplantation of the gland to a distant site removes it from the sphere of its normal stimulus. This idea was tested in experiments made in collaboration with Dr. Dora Jacobsohn (Harris and Jacobsohn, 1951), in which it was shown that pituitary grafts placed in a site where they became revascularized by the hypophyseal portal system will show normal function, but if placed elsewhere show very little, if any, activity.

At the present time it may be taken as established that the hypophyseal portal vessels are intimately concerned with the maintenance and control of anterior pituitary activity. The exact mechanism by which they perform this function is unknown. It seems more likely that they transmit a specific humoral substance liberated into the primary plexus by nerve fibres of the hypothalamus, than that a control exists dependent on total pituitary blood supply.

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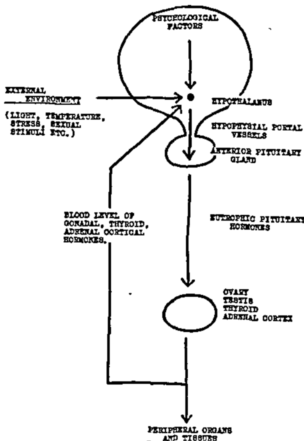


Fig. 1. Diagram to illustrate

humoral factors (blood level of œstrogens, adrenal cortical hormones, etc). The total effect of such influences is transmitted via the hypophyseal portal vessels of the pituitary stalk to the anterior pituitary gland. It would seem likely that the "feed back" of hormones secreted from the target endocrines (gonads, thyroid, adrenal cortex) to the central nervous system is responsible for the behavioural patterns of various endocrine states. It is possible that the hypothalamus is responsible not only for adjusting anterior pituitary secretion according to the blood level of these substances, but may also play an initiating rôle in the overt behavioural patterns such as occur, for example, in the state of œstrus.

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FUNCTIONAL HYPOPHYSEAL GRAFTS

G. W. HARRIS and DORA JACOBSOHN

WHETHER the anterior pituitary gland retains its functional capacity after complete separation from the brain is a question which has received much attention. Many workers have studied the viability and function of pituitary glands transplanted into different sites in hypophysectomized animals. The function of these transplants was generally found to be slight or absent, a result which might be due, at least in part, to factors inherent in the transplantation. Another technique,

Harris, 1948). After transection of the pituitary stalk in rabbits and rats, Westman, Jacobsohn and Hillarp (1943) found that the anterior pituitary gland, which had been completely separated from the brain, ceased to function. If, however, any connection between the pars tuberalis and the pars distalis remained, then anterior lobe function persisted. From these observations it was concluded that cerebral control as effected by the pars distalis-pars tuberalis connection is essential for any functional activity of the gland. Harris (1950) also transected the pituitary stalk in rats, and concluded that anterior lobe function is dependent on vascularization of the gland by the hypophyseal portal vessels. These vessels pass through the pars tuberalis, and it was suggested that they are responsible for transmitting a humoral stimulus from the hypothalamus to the anterior pituitary gland which maintains and controls the activity of this gland.

The functional activity of pituitary grafts was now reinvestigated (Harris and Jacobsohn, 1951). Particular attention was paid to the possibilities that vascularization of

appropriately placed grafts might be obtained from the hypophyseal portal vessels, and if so whether such vascularization was essential for any function of such grafts. This work forms the subject of the present paper.

As recipients of the grafts, adult female rats from an inbred strain were used. The donors were the recipient's own new-born young, 2–10 days old. The use of such donors for hypophyseal transplants had given the best takes in a previous investigation by Westman and Jacobsohn (1940). The donors' hypophyseal tissue was grafted into hypophysectomized rats: (1) under the temporal lobe of the brain or (2) under the median eminence of the tuber cinereum. Only the latter grafts could reasonably be expected to receive a supply from the hypophyseal portal vessels.

The hypophysectomy and the grafting were performed in a one stage operation. One of us (D.J.) hypophysectomized the rats by the parapharyngeal route, and the other (G.W.H.) performed the grafting operation using the temporal approach as described for stalk transection by Harris (1950). The donor rats were killed and their pituitary glands dissected out immediately before placing in the recipients. Some of the grafted animals were observed for several months, but most of them were killed after six weeks' observation. During this time the reproductive functions were studied. At the end of an experiment the vascular system was perfused with indian ink, and the head fixed and decalcified. A block containing hypothalamus, pituitary gland and base of skull was embedded in paraffin wax and cut into serial sections: one section 100μ, six sections 10μ, and four sections 5μ. By this method the distribution of the vessels as well as the microscopic picture of the grafts and the operation field could be studied. As indicators of any function of the grafts the weight and microscopic appearance of the ovaries and adrenal glands, and in some cases the microscopic appearance of the thyroid, were used.

Fig. 1 shows typical examples taken from a group of 10 experiments with grafts placed under the temporal lobe of the



A



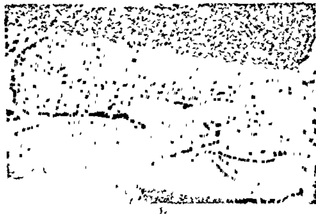
B



C



D



E

Fig. 1

A. Horizontal section through a transplant under the temporal lobe of the brain. Unstained. 100μ × 52.

B. Adjacent section of the specimen shown in A. Hemat.-eosin. 10μ × 112.

C. Midline sagittal section through hypothalamus and graft under the median eminence supplied by vessels originating in the primary plexus of the hypophyseal portal system. Unstained. 100μ × 52.

D. Adjacent section of the specimen shown in C. The graft consists of anterior lobe tissue. Hemat.-eosin. 10μ × 112.

E. Horizontal section through grafts of the recipient's own young placed under the median eminence. Hemat.-eosin. 5μ × 57.

brain, and of 12 with grafts under the median eminence. In all these 22 experiments the grafted pituitary glands were well vascularized and viable. Only those grafts which were placed under the median eminence were supplied by the hypophyseal portal vessels, and only these grafts showed good cellular differentiation.

The findings related to the functional activity of the grafts were as follows. Daily vaginal smears revealed the occurrence of œstrous cycles in all of the 12 hypophysectomized recipients with grafts from their own young placed under the median eminence. None of the 10 recipients with similar grafts placed under the temporal lobe came into œstrus. Grafts placed under the temporal lobe of the brain then, on the evidence of the vaginal smears, showed a lack of gonadotrophic secretion. This conclusion was substantiated at the end of the experiment when the ovaries were examined microscopically and found to be atrophic. The mean weight of these structures was 27·3 mg. ±2·08 (10). The adrenal glands were also atrophic, though not as completely as in hypophysectomized rats. The mean weight of both adrenals was 20·7 mg. [illegible]

marked. These observations agree with those made previously by Westman and Jacobsohn (1940) on hypophysectomized rats with hypophyseal transplants from their own young placed in the anterior chamber of the eye.

Strikingly different from these findings were those made on the hypophysectomized recipients with grafts placed under the median eminence. The 12 recipients of this group which, as noted previously, had œstrous cycles, were placed together with normal males. Six of these animals became pregnant and delivered living, normally developed young at term. One had an abortion and the other five failed to become pregnant. After parturition the maternal mammary glands of the six rats were found to be well developed, the alveoli and ducts being distended with secretion. In spite of vigorous

suckling, however, the young did not obtain the milk present in the mammary glands and died from starvation. It was found that this could be prevented by subcutaneous injections of oxytocin (100–300 mU.) into the maternal rat. After such an injection a copious transfer of milk occurred from the mother to the litter, a process which could be observed through the transparent abdominal walls of the young. By this procedure, repeated three times a day, one of the litters was raised and has now produced a second generation. These observations indicated that the grafted anterior lobe tissue was functional whilst the posterior lobe tissue was not. The post-mortem findings confirmed this conclusion. Microscopically the ovaries and adrenals presented a normal picture. The mean weight of both ovaries was 53 mg. $\pm 3 \cdot 49$ (12) and of both adrenals $41 \cdot 7$ mg. $\pm 1 \cdot 16$ (12). The thyroid, examined microscopically in 9 cases, appeared normal.

The experiments presented here show that the functional activity of grafted anterior lobe tissue depends upon the anatomical site of the graft. Experiments with hypophyseal transplants under the temporal lobe of the brain or into the anterior chamber of the eye show that good vascularization, irrespective of the source of the blood, is not sufficient to render anterior lobe tissue functional. Some additional, specific factor is obviously necessary and this factor is present in those experiments in which the graft is placed under the median eminence. When placed in this site the graft becomes revascularized by vessels regenerated from the primary plexus of the hypophyseal portal system which pass through the pars tuberalis to the rest of the grafted tissue.

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DISCUSSION

Vazquez-Lopez: I would like to raise some objections to Dr. Harris's paper. There are some technical details in the crucial experiment of Harris and his co-workers that are capable of a completely different explanation in relation to the transmission of the stimulus. I agree that the portal vessels may have some very important function, but the [illegible]

electrically you get a gonadotrophic response that you don't get when you stimulate the anterior lobe itself. The important point here is the spread of the electrical stimuli. I think that the spread in the experi- [illegible]

volume of anterior lobe

Harris: That is a point we have considered. There are two facts, however, that seem to be important in this connection. Firstly, the [illegible]

ovarian response if stimulated for as long as 450 minutes. Secondly, [illegible]

result in ovulation—but it does not

Markee: In our experiments I think that in five of them we had the electrodes far enough apart so that a considerable amount of the anterior lobe must have been stimulated.

VAZQUEZ- stalk a find th But if t

HARRIS. The ... regenerate the nerves ... the function reappears. considered a lot. The question of nerve regeneration is a point which ... that there ... I think all that can ... the nerves will regenerate too. will regene ... from the h ... would expe ... clear for the ... not to regene... the same ...

VAZQU ... part of ...

VAZQUEZ-LOPEZ these nerves in the ... and think they are probably demonstrable in the transplant ... found

KAHLSON. When you stimulate ... thus ... migh ... I wor ... poster

HARRIS. If you ... secretomotor nerves ... direct stimulation of the either c ... fibres a ... as meas ... the water output or on uterine contraction. If your electrode is right in the posterior lobe you get a response that is almost equal, if not quite so large. That might be for one or two reasons, but it is very little different from the response where you stimulate the whole tract at a site further forward. On those grounds, if there is a comparable nerve supply between the neurohypophysis and the adenohypophysis, you would certainly expect a response by stimulating with the electrode in the pars distalis ... from the posterior lobe,

KAHLSON: From the answer given I think the experiments support the view that nerve fibres in the anterior lobe, if there are such (the lecture by Dr. Vazquez-Lopez yesterday has convinced us that there are such fibres) might not be secretory.

VAZQUEZ-LOPEZ: I don't agree ...

... of nerves. Practically

anywhere you put the electrode in the posterior lobe you are going to excite a considerable number of nerve fibres, so when you stimulate in the posterior lobe it is practically the same as when you stimulate in the hypothalamic tract.

KAHLSON Physiologists will agree that nerve terminals are easily stimulated, and I should be surprised if the spread of the stimulus, using this technique, is not adequate to activate a considerable proportion of nerves in the anterior lobe

VOGT May I ask Dr. de Groot a question about one slide he showed us. Is it a regular finding, or was it just accidental that in the slide he showed, in the hypophysectomized animals the basic level of lymphocyte was low?

DE GROOT: I think we found regularly that the first few days after hypophysectomy the lymphocyte level was lower than in normal animals, but in control experiments the hypophysectomized rabbits reacted to ACTH injections as did normals.

VOGT. You mean the *percentage* fall was the same?

DE GROOT: Yes

DESCLIN: I should like to say a word about the pituitary grafts. Years ago we studied pituitary grafts in the kidney and the influence of œstrogen on the structure of the pituitary graft, and we found that the changes which result from the œstrogen administration take place in the graft as well as in the pituitary in its normal location. If those [illegible]

[illegible] administration Mucification of the vagina and lobuloalveolar development of the mammary gland are sometimes present.

[illegible]

complete stalk transection produces some effects which you do not get [illegible]

[illegible]

hypothalamus. It seems moreover that œstrogens can act directly on

VAZQUEZ-LOPEZ: With regard to the experiments on the section of the stalk and the transplants in the tuber cinereum: if you cut the stalk you find that the portal vessels regenerate, and the function reappears. But if the portal vessels regenerate the nerves will regenerate too.

HARRIS: The question of nerve regeneration is a point which we have considered a lot. I think all that can be said at the moment is this, that there is no evidence at all that nerve fibres into the posterior lobe will regenerate. And on the assumption that the supposed nerve fibres from the hypothalamus to the anterior lobe are the same type, one would expect those also not to regenerate. That point has been made clear for the posterior lobe by Ranson and his workers and many others.

VAZQUEZ-LOPEZ: I agree that in the posterior lobe, which is after all a part of the central nervous system, you perhaps don't expect a regeneration. But when you cut the stalk, naturally you cut this tract of nerve fibres, which are not any more in the central nervous system, fibres which run alongside vascular structures and which are morphologically exactly the same as any peripheral nerve fibre, which will regenerate.

HARRIS: We considered staining these transplants to see if there were nerve fibres present, but since we couldn't find any nerve fibres at all in the normal pars distalis, it made this a rather doubtful procedure.

VAZQUEZ-LOPEZ This, of course, is where we disagree. I have found these nerves in the normal animal and think they are probably demonstrable in the transplant

KARLSON. When you stimulate with electrodes in the anterior lobe, thus activating nerve terminals, and you get no secretory response, this might indicate that there are no secretory nerves to the anterior lobe. I wonder what results have been recorded on direct stimulation of the posterior lobe where secretomotor nerves are known to be present.

HARRIS: If you stimulate the supraoptic tract of the posterior lobe, either on the median eminence or the infundibular stem where the nerve fibres are concentrated, you get a response from the posterior lobe, as measured on the water output or on uterine contraction. If your electrode is right in the posterior lobe you get a response that is almost equal, if not quite so large. That might be for one or two reasons, but it is very little different from the response where you stimulate the whole tract at a site further forward. On those grounds, if there is a comparable nerve supply between the neurohypophysis and the adenohypophysis, you would certainly expect a response by stimulating with the electrode in the pars distalis.

KARLSON: From the answer given I think the experiments support the view that nerve fibres in the anterior lobe, if there are such (the lecture by Dr. Vazquez-Lopez yesterday has convinced us that there are such fibres) might not be secretory

VAZQUEZ-LOPEZ. I don't agree for this reason: the function of the nerves to the posterior lobe may not be secretory at all. If the posterior lobe is, as I believe it is, a receptor organ, whatever the stimuli that you prepare, the stimuli will be transmitted to the hypothalamus. But in any case, the conditions in the posterior lobe are completely different from the anterior lobe. The posterior lobe is full of nerves. Practically

function normally, so I don't think the total blood supply is the crucial point in these experiments, but rather the function of the glandular tissue depends on whether it is vascularized solely by systemic vessels or by the hypophyseal portal vessels as well

BROBECK: Is the graft smaller?

JACOBSOHN: They are the same size. I have also studied the vessels in rabbits with the hypophyseal stalk transected, and the hypophysis which is completely separated from the brain and has no supply from the portal vessels is very richly vascularized, but has no function.

the pituitary as well as on the hypothalamus, as is evident from what Dr. Desclin told us. It looks then as though some, at any rate, of the secretions of the [illegible]

organs, the pituitary itself becomes hypoactive; but supply some of these secretions artificially, and some at least of the pituitary functions will then carry on.

HARRIS I think it isn't possible to exclude a direct action of the target gland hormones back on the pituitary itself, but I think there is considerable evidence that they do act back on the hypothalamus. The idea of the œstrus cycle that Dr. Everett put forward would involve the hypothalamus in the œstrus cycle, i.e., the ovarian hormones are reacting back in reciprocal fashion on the hypothalamus, and via that on to the pituitary From the point of view of the grafts there is the point that the pituitary tissue of male rats grafted on the median eminence of female rats will maintain normal œstrus cycles in the hosts, and these rats can become pregnant Then if you take the pituitary tissue of new-born rats and place them on the median eminence of their hypophysectomized mothers, œstrus cycles may start within a matter of 10 days, so that the animal is having cycles from pituitary tissue that is only actually 12 days old. In other words, the pituitary tissue seems to be very plastic tissue which is open to hypothalamic drive. This is a little away from your point, but I think that the hypothalamus is the driver.

SWYER. Yes, I wouldn't deny that, but what I am suggesting is that, for example, œstrogen can make the pituitary, even when it's not connected to the hypothalamus, produce LH. That is what Dr Desclin said, and that is the sort of effect I was thinking of.

BROBECK: With regard to the blood supply to the anterior pituitary: what is the evidence that it is the portal part of the blood supply rather than the total blood supply that is essential?

HARRIS: In rats when the pituitary stalk is cut by the temporal route one usually sees some fibrosis develop in the centre of the pars distalis However, in a series of animals examined some weeks after stalk section we could find no correlation between the return of reproductive functions and the amount of anterior pituitary tissue present in the sella turcica, or with the total vascularization of this tissue (as judged by indian ink injections). There was, however, a correlation between the return of reproductive functions and regeneration of the portal blood vessels across the site of stalk section. And then regarding the grafts. As far as one can see with injection, the temporal lobe [illegible]

one hand the graft is [illegible]

activated in more than one way, the nature of the required pathway or mediator being determined by the nature of the stress applied. The following two experiments were done in order to test this hypothesis.

Experiment A

Comparative Response of the Eosinophils to Neurotropic and to Systemic Stimuli after Hypophyseal Transplantation

Exposure of rats to intense sound or light was shown in this laboratory to elicit a rapid and intense discharge of ACTH, as evidenced by the depletion of the adrenal ascorbic acid content (Fortier, 1950*a*, *b*). Similar results were reported by the Cambridge group with the emotional stress of immobilization which induced a marked lymphopenia in the rabbit (Colfer *et al.*, 1950). Administration of cortisone (Fortier *et al.*, 1951*a*, *b*), adrenodemedullation or the use of Dibenamine, an adrenolytic agent (Fortier, 1951*a*, *b*), proved, in our hands, equally ineffective in preventing the sound-induced adrenal ascorbic acid depletion, which was, however, suppressed by transplantation of the pituitary from its normal site into the anterior chamber of the eye (Fortier, 1951*a*, *b*). Likewise, as shown by the Cambridge workers, the emotionally induced lymphopenia of the rabbit, persisting after denervation of the adrenal gland (Colfer *et al.*, 1950), "was abolished by lesions in the pars tuberalis and, in most cases, was abolished or diminished by transverse lesions in the posterior region of the tuber cinereum or in the mammillary body" (De Groot and Harris, 1950). The corticotrophic effect of such neurotropic stimuli as sound and immobilization was apparently dependent on the integrity of these very hypothalamo-hypophyseal pathways whose suppression proved compatible with the release of ACTH in response to systemic stimuli (cold, histamine, adrenaline) (Cheng *et al.*, 1949*b*, Fortier and Selye, 1949; Fortier, 1950*b*; McDermott *et al.*, 1950*a*, *b*) Hence, the following experiment was planned to compare the corticotrophic effect of the two varieties of stimuli, as judged by the

STUDIES ON THE CONTROL OF ACTH RELEASE BY MEANS OF HYPOPHYSEAL TRANSPLANTS†

*CLAUDE FORTIER**

THE search for a common mediator of the stress-induced release of ACTH from the adenohypophysis has led so far to an apparent deadlock. Of the three mechanisms independently shown to play a rôle in the activation of the pituitary corticotrophic function (Sayers, 1950*a*), none has proved essential for the release of ACTH under all conditions of stress.

Administration of cortical hormones to prevent the lowering of their venous titre (Moya and Selye, 1948, Sayers, 1949, 1950*b*; Gershberg *et al.*, 1950, Fortier *et al.*. 1951*a*, *b*), exclusion of the sympatho-adrenal system through complete sympathectomy (Hume and Wittenstein, 1950, Recant *et al.*, 1950), denervation of the adrenal gland (Vogt, 1947; Colfer *et al.*, 1950), adrenodemedullation (Gershberg *et al.*, 1950; Gordon, 1950; Long, 1950; Recant *et al.*, 1950; Fortier, 1951*a*, *b*; Nasmyth, 1951), the use of adrenolytic agents (Tepperman and Bogardus, 1948, Seifter *et al.*, 1949; Wiedeman and Lewis, 1949; Gershberg *et al.*, 1950, Paschkis *et al.*, 1950, Ronzoni and Reichlin, 1950, Sayers, 1950*b*; Fortier, 1951*a*, *b*; Nasmyth, 1951), deafferentiation of the hypophysis through pituitary-stalk section (Uotila, 1939; Keller and Breckenridge, 1947; Cheng *et al.*, 1949*a*; Fortier and Selye, 1949; De G[illegible] Harris, 1950, Recant *et al.*, 1950, Selye and Fortier, [illegible] 1949*b*; [illegible] *et al.*, 1950*a*, *b*; Selye and Fortier, 1950*a*, *b*), equally failed to prevent ACTH release in response to various stressing procedures.

This is compatible with the view that the pituitary may be

*Presented at the Colloquium on behalf of Dr. Fortier, absent through illness, by Dr. Paola S. Timiras.

†This work was done during the tenure of an American Heart Association

sub-groups, each made up of one half of the experimental group, underwent respectively either the cold or the histamine test. The adrenaline test was repeated, in a final stage, on six animals whose graft-containing eye had been enucleated on the previous day.

Eosinophils. Immediately before and three hours after initiation of the stimulus, direct counts of blood eosinophils were made on freely flowing samples of tail blood by the selective method of Randolph (1949). Uniform diluting pipettes were utilized. The mixing of the blood was standardized by the use of a Burton pipette-shaker and the eosinophils were counted on both sides of a Levy-Neubauer counting chamber of 0·2 mm. depth. A rather wide range of variation was observed in the initial levels of the eosinophils in both intact (100–275/mm.^{3}) and pituitary-grafted (75–450/mm.^{3}) animals. However, the high correlation coefficient observed by the Yale workers between the initial levels and the absolute falls of these elements "makes it statistically permissible to employ percentage fall as an accurate index of change" (McDermott *et al.*, 1950*b*). Our results have been expressed in this way.

Histology. The animals were killed by bleeding at the end of the experiment. The adrenals and testes were fixed in Bouin's fluid prior to weighing. The graft-containing eye (or eyes) was removed, its posterior half cut away and the remaining portion fixed in Bouin-Hollande extemporaneously mixed with sublimate ($HgCl_2$, 10 per cent). The pituitaries of the normal controls and the sellar regions of the hypophysectomized animals were similarly fixed, the latter prior to decalcification This material, sectioned at 7μ, was stained with Masson's modified trichrome. Completeness of the hypophysectomy, first checked at autopsy with a 5 × magnifying glass, was later ascertained by microscopic examination of serial sections of the sellar region. Pituitary remnants were discovered in four cases and the original experimental group was reduced to the 18 animals which are included in this report.

fall of the circulating blood eosinophils, after separating the *adenohypophysis from the hypothalamic centres* and transplanting it into the anterior chamber of the eye.

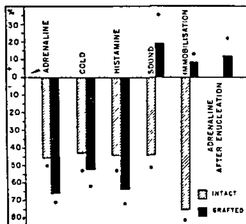
Methods

Preparation of the Animals. Male piebald rats of an average initial weight of 100–120 g. were used. Adenohypophyseal transplants were aseptically introduced, under light ether anæsthesia, into the anterior chamber of either one or both eyes, *according to a previously described technique* (Fortier and Selye, 1949). Animals of the same sex and weight served as donors. Two days later, hypophysectomy was performed by the standard parapharyngeal approach. As a preventive measure, on the day of the operation and at weekly intervals for the following six weeks, penicillin in oil was injected subcutaneously in a dose of 50,000 units. The animals were kept, for the duration of the experiment, at a *constant room temperature of 28° to 30°C. They were given* a 5 per cent glucose solution to drink and maintained on a diet of dry pablum.

Stimuli. Between 45 to 55 days postoperatively the tests were initiated on 22 animals whose pituitary transplants showed adequate morphological take, and on eleven intact controls. They were repeated at weekly intervals with different stimuli including adrenaline (epinephrine HCl, 0·02 mg./100 g. dissolved in 0·2 ml. of 0·9 per cent NaCl and injected subcutaneously), histamine acid phosphate (1 mg./100 g. also dissolved in 0·2 ml. of 0·9 per cent NaCl and administered intraperitoneally), auditory stimulation (30 minutes of exposure to sound generated by a type 2, 2 h.p., 110 v., 25 amps., Federal Siren), cold (3 hours of exposure at 0° to 2°C.) and immobilization (3 hours). The latter stress was induced by firmly wrapping up the animal with adhesive [illegible] way as to allow for ample [illegible] nge in position (Mann and [illegible] were successively exposed to the adrenaline, sound and immobilization stimuli. Two

fewer basophils, and poorly staining sparsely granulated chromophil cells whose identity could not be ascertained.

Body and Organ Weights. As can be seen from Table I, the transplants had very little if any effect on body growth and evidenced a markedly deficient gonadotrophic activity, as judged by the prevalent testicular atrophy. By contrast, the



adrenal glands showed a much smaller degree of atrophy and were, as a whole, adequately maintained.

Eosinophils. The response afforded by the circulating eosinophils was well defined (Fig. 1). Adrenaline, cold and histamine, the so-called systemic stimuli, induced a marked fall of these elements in both intact and grafted animals. Sound and immobilization, the neurotropic agents, produced a comparable eosinopenia in the controls but none in the pituitary-grafted animals. No fall of the eosinophils was elicited by adrenaline following removal of the graft-bearing eye.

Results

Histology of the Transplants. The ocular implants in most cases were richly vascularized and presented various degrees of invasion by a dense connective tissue stroma. Islets of viable glandular tissue showing typical nest formations were found, however, in all of them. As in previous studies, the trichrome staining showed a great predominance of chromophobic elements interspersed with rare acidophils,

Table I

EFFECT OF PITUITARY TRANSPLANTATION ON BODY GROWTH, ADRENAL AND TESTIS WEIGHT

Groups	*No*	*Increase in weight*	*Adrenal mg /100 b w*	*Testis mg /100 b w.*
Controls	A	+200	17	1130
	B	+188	18	971
	C	+210	19	767
	D			
	E	+200	13	991
	F	+248	14	964
	G	+218	22	1022
	H	+248	19	1039
	I	+190	19	1051
	J	+146	20	1335
	K	+144	14	1055
	Average	+199±10	18 5±1	1028±46
Bearers of Pituitary Transplants	A	+28	25	649
	B	+10	16	185
	C	+14	11	285
	D	+ 8	10	144
	E	+44	31	268
	F	−22	12	516
	G			
	H	−11	14	221
	I	− 4	12	235
	J	+10	16	218
	K	−20	15	263
	L	− 2	14	201
	M	+26	10	221
	N	+ 4	19	777
	O	+18	12	214
	P	+ 2	11	180
	Q	+12	15	706
	R	+ 6	19	205
	Average	+5±4	15 4±1 3	318±50

(De Groot and Harris, 1950). The latter would be relayed to the adenohypophysis via the hypophyseal portal vessels by means of an unknown neuro-humoral agent (Harris, 1948; De Groot and Harris, 1950) The enhancement of the eserine-induced adrenal ascorbic acid depletion by simultaneous administration of atropine would seem to preclude the possibility of this agent being of a cholinergic nature (Dordoni and Fortier, 1950, 1951).

Even though dissociated, under our experimental conditions, the above two mechanisms should not be considered as mutually exclusive in relation to a given stimulus, and it is logical to assume that they are concurrently called into play under the many stress conditions which partake of both nervous and systemic components.

Experiment B

Response of the Eosinophils to Direct Stimulation of Hypophyseal Transplants

A series of brilliant investigations conducted by Long and co-workers (Gershberg *et al.*, 1950, McDermott *et al.*, 1950*a*, *b*) has led to the concept that "the mechanism of control of the secretion of the adrenal cortex, through the release of adrenocorticotrophic hormone from the pituitary, apparently consists of two phases which may be independent or sequential, depending on the conditions of stress encountered by the organism. These phases have been labelled autonomic and metabolic, terms which seem descriptive of their physiological characteristics. The first or autonomic phase depends on the reflex secretion of epinephrine which directly activates the anterior pituitary, while the second or metabolic phase is based upon the rate of utilization of adrenal cortical hormones within the organism" (McDermott *et al*, 1950*b*). The reflex secretion of adrenaline and, within physiological or near physiological limits (Fortier *et al.*, 1951*a*, *b*), the level of circulating cortical hormones are admittedly related to the regulation of ACTH release (Sayers, 1950*a*). It has already

Discussion

The functional activity of the transplants is of obvious importance in an experiment of this type and forms the prerequisite for its interpretation. From a histophysiological standpoint, the increased chromophobe/chromophil cell ratio, which has proved in this as well as in previous studies a constant feature of the transplanted pituitary (Cheng *et al.*, 1949*b*; Fortier and Selye, 1949; Fortier, 1950*b*, 1951*b*), strongly suggests a loss of function which is further borne out by the testicular atrophy and arrested growth of the animals. All the more evident is the repeatedly observed dissociation in pituitary transplants between growth and gonadotrophic activities on the one hand, and the corticotrophic function on the other (Cheng *et al.*, 1949*b*; McDermott *et al.*, 1950*a*, *b*; Fortier, 1951*b*). The latter was well preserved, as judged by the partial maintenance of the adrenals, the stress-induced eosinopenia and its prevention by removal of the graft-containing eye.

Our results, so far in agreement with previous similar studies, point however to a definite selectivity in the corticotrophic response to stress of the transplanted pituitary. The lack of response to neurotropic stimuli of otherwise reactive transplants, supports our original contention regarding the stimulus specificity of the pathways involved in corticotrophic activation. At least two different mechanisms of ACTH release are suggested by the above results: the first, induced by so-called systemic agents, would imply the direct activation of the pituitary by one or several humoral agents, the nature of which remains the object of interesting speculations (Gershberg *et al.*, 1950, Long, 1950; McDermott *et al.*, 1950*b*; Sayers, 1950*a*, *b*; Vogt, 1950); while the other, brought forth by neurotropic stimuli, would require the participation of the hypothalamo-hypophyseal pathways.

The brilliant investigations of Harris and co-workers, with the stereotaxic stimulation method, point to the posterior region of the tuber cinereum and the mamillary body as the most probable sites for the integration of the nerve impulses

by the same authors to induce the release of ACTH. The dosages of dibenamine (0·03 mg./100g.) and histamine (0·01 mg./100g.) were correspondingly adjusted, on the basis of experiments done in this laboratory. Cold was locally applied to the transplants by freezing the contents of the anterior chamber by means of an ethyl-chloride spray directed towards the cornea for 10 seconds. The stimuli which induced a fall of the eosinophils were repeated, after an interval of four days,

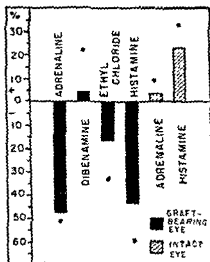


FIG. 2 Response of the eosinophils to direct stimulation of *hypophyseal transplants.*

on the intact eye. The blood eosinophils were counted three hours after stimulation.

Results

Of the four agents tried, and at the concentrations used, two, adrenaline and histamine, induced a pronounced fall of the eosinophils when applied to the graft-containing eye (Fig. 2). Localized cold and dibenamine were ineffective in this respect, and so were adrenaline and histamine when injected into the intact eye.

been pointed out, however, that interference with either process is, under certain experimental conditions, compatible with the stress-induced release of ACTH and that, furthermore, both mechanisms fail to activate the pituitary in response to neurotropic stimuli (Experiment A). Likewise the direct ACTH-releasing effect of adrenaline locally applied to pituitary grafts (McDermott *et al.*, 1950*a*, *b*) does not rule out the possible participation of other similarly acting agents in the regulation of the corticotrophic function. *With the object of testing this possibility, direct stimulation of hypophyseal transplants with various agents (adrenaline, cold, histamine, dibenamine) was attempted in the following experiment; the fall in circulating blood eosinophils being used again as a criterion of ACTH release.*

Methods

The tests were initiated between 75–85 days after hypophyseal transplantation on eight animals which had been used in the preceding experiment. The stimuli were locally applied to the graft-containing eyes under light nembutal anæsthesia (4 mg./100g.), an interval of four days being allowed to elapse between successive tests. The chemical agents (adrenaline HCl, dibenamine HCl and histamine acid phosphate) were adjusted with 0·9 per cent NaCl to a volume of 0·05 ml. and administered through sub-conjunctival injections distributed between four equidistant sites to accelerate absorption. This mode of administration was adopted in preference to the direct application procedure described by McDermott *et al.* (1950*a*, *b*) Less traumatizing for the eye-ball, it prevents the loss of administered fluid and the danger of hæmorrhage into the anterior chamber encountered with the latter procedure, while allowing for repeated tests and ensuring a rapid and localized absorption, as evidenced by the development of unilateral mydriasis following adrenaline administration. The amount of adrenaline injected (0·004 mg./100g.) was similar to the dosage reported by McDermott *et al.* (1950*a*, *b*) and corresponds to 1/100 of the dose parenterally administered

by the same authors to induce the release of ACTH. The dosages of dibenamine (0·03 mg./100g.) and histamine (0·01 mg./100g.) were correspondingly adjusted, on the basis of experiments done in this laboratory. Cold was locally applied to the transplants by freezing the contents of the anterior chamber by means of an ethyl-chloride spray directed towards the cornea for 10 seconds. The stimuli which induced a fall of the eosinophils were repeated, after an interval of four days,

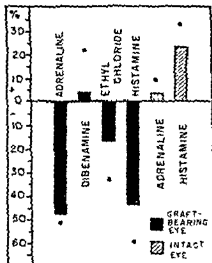


FIG 2 Response of the eosinophils to direct stimulation of hypophyseal transplants

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Discussion and Conclusions

The above results throw serious doubt on the response-specificity of the pituitary for a given humoral mediator of ACTH release. While confirming the Yale workers' contention of a direct effect of adrenaline on the gland, they point to a similar action of histamine. However, like adrenaline or alterations in the level of cortical hormones, the latter agent is not essential for the stress-induced increase in ACTH secretion; Phenergan (1-dimethyl-amino-2'methyl, 1'ethyl-n-dibenzo-p-thiazine hydrochloride, also known as 3277 R.P.), a powerful antihistaminic drug, fails to prevent in the rat the adrenal ascorbic acid depletion consequent to cold exposure (Fortier and Guillemin, unpublished). If specific pathways (hypothalamo-hypophyseal connections) are apparently required for the activation of the pituitary in response to neurotropic stimuli (Experiment A), it is suggested, on the basis of present evidence, that the corticotrophic effect of systemic stimuli is mediated through various humoral changes induced by stress (Selye, 1951) and acting either independently or concurrently on the hypophysis to elicit the release of ACTH.

Summary

The ACTH response to systemic (adrenaline, cold, histamine) and to neurotropic (sound, immobilization) stimuli was compared in rats, following separation of the adenohypophysis from the hypothalamic centres through homotransplantation of the gland into the anterior chamber of the eye. These animals, along with normal controls, were stimulated at weekly intervals and the fall of the circulating blood eosinophils used as an index of ACTH release. Adrenaline, cold and histamine brought about a definite eosinopenia in both normal and grafted animals. Sound and immobilization induced a marked fall of the eosinophils in the intact but none in the grafted animals.

In a complementary experiment, direct stimulation of the hypophyseal transplants with cold, adrenaline, dibenamine

and histamine was attempted. Sub-conjunctival injections of adrenaline and histamine into the graft-containing eye resulted in a pronounced fall of the eosinophils. Localized cold (ethyl-chloride spraying of the cornea) and dibenamine were ineffective in that respect, and so were adrenaline and histamine when injected into the intact eye. It is suggested, on the basis of present evidence, that the hypothalamo-hypophyseal connections are required for the activation of the pituitary in response to neurotropic (nervous or emotional), stimuli, while the corticotrophic effect of systemic stimuli is mediated through various humoral changes induced by stress and acting either independently or concurrently on the pituitary to elicit the release of ACTH.

Acknowledgements

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DISCUSSION

... of the anterior lobe to the
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report. And, as I shall show you this afternoon, our experiments on the

cortical function.

in the controls.

Kahlson: It seems difficult to assess the function of a transplant put into the eye chamber unless we know how great a proportion of the hormone which might be liberated passes the barriers between the transplant and the general circulation.

Long: Yes, but isn't it evident if one accepts the fall in eosinophils as a measure of adrenal cortical secretion that some ACTH has penetrated that barrier and reached the adrenal?

Vogt: The experiment suggested by Dr. Kahlson will only give the right information if it is correct to assume that there is the same barrier in an eye that has been operated on and has scar tissue as in a normal

Smelser: A pituitary graft on the iris would probably give up its secretion to the aqueous humour, and once there it would pass to the canal of Schlemm and there pass out of the eye by diffusion and flow.

Kahlson: Yes, but isn't the rate of flow through the channel rather slow?

Smelser: Recent work has shown that it is remarkably fast. Experiments by Maurice and Davson in London and by Kinsey at Harvard

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DISCUSSION

LONG: Our results with the transplantation of the anterior lobe to the eye, as far as the maintenance of the adrenal weight is concerned, the failure to maintain the weight of the testicles or to observe any significant amount of growth, entirely agree with those in Dr. Fortier's

THE RÔLE OF EPINEPHRINE IN THE SECRETION OF THE ADRENAL CORTEX

C. N. H. LONG

THERE would appear to be little doubt that epinephrine is a potent stimulus to the secretion of adrenocorticotrophic hormone (ACTH) by the anterior pituitary. The conclusion has been reached by several groups of investigators, all of whom have employed different methods for the detection of an increased rate of secretion. By direct measurement of the output of adrenal cortical hormone after the intravenous injection of epinephrine in dogs, Vogt (1944) found that the rate of secretion rose rapidly and continued for some time after quantities of epinephrine that may be regarded as within the physiological rate of secretion of the adrenal medulla. Although at first she was inclined to the view that the effect of epinephrine on the adrenal cortex was a direct one, in a later paper with Pickford (Pickford and Vogt, 1951) she concluded that an increased secretion of adrenal cortical hormones is only observed if the hypophysis is present. In other words, the effect of epinephrine on adrenal cortical secretion is by a preliminary activation of ACTH secretion.

By the use of the adrenal cholesterol or ascorbic acid depletion method, Long and Fry (1945), and later Gershberg, Long and Fry (1950), also concluded that epinephrine enhanced adrenal cortical secretion by augmentation of ACTH secretion. They observed in normal rats that the subcutaneous, intravenous or intramuscular injection of epinephrine in quantities of the order of a few micrograms was followed within an hour by a well marked fall in both the cholesterol and ascorbic acid content of the gland. It should be noted that the depletion of either the adrenal cholesterol or ascorbic acid is a measure of the blood level of ACTH,

show that approximately 40 to 50 mm.^{3} of total aqueous leave the eye per minute in the experimental animal. These experiments were done

recall, there was no evidence of any significant maintenance of the thyroid.

TIMIRAS. I think Dr. Fortier didn't mention the thyroid because his results lacked consistency in that respect. However, as far as I can remember, they somewhat paralleled his observations in regard to the gonads. A more extensive study would be required to reach definite conclusions.

maintained with œstrogens (Bourne and Zuckerman, 1940). In hypophysectomized pigeons Miller and Riddle (1939) have reported that the size of the adrenal is increased by the administration of thyroid, insulin or formaldehyde.

The very large number of circumstances that have been shown to be associated with an increased rate of adrenal cortical secretion may be presumed to bring about that effect by stimulating the secretion of ACTH. Since many of these same circumstances are also those that have been known for some time to be associated with activation of elements of the autonomic system and release of epinephrine, we have suggested (Long, 1946) that the immediate release of ACTH from the anterior lobe is a consequence of the combined nervous and humoral response to conditions which threaten the existence of the organism. You will recognize that such a hypothesis is in effect an extension of the views previously advanced by Cannon and his associates concerning the rôle of the autonomic nervous system and epinephrine in the preservation of the organism.

The Nature of the Effect of Epinephrine on Adrenal Cortical Secretion

The effects of liberation of epinephrine into the circulation are so widespread and diverse, mimicking as they do those of generalized sympathetic activity, that it is no easy matter to determine the exact manner by which this hormone evokes the secretion of ACTH

Some of the possible ways may be considered. (*a*) It has been suggested by Sayers (1950) and by Selye (1950) that the effect of epinephrine is non-specific. Although Selye has not defined exactly what is meant by a non-specific effect of epinephrine, a physiological agent that heretofore has been regarded as highly specific in its action, Sayers has suggested that it stimulates ACTH secretion by its capacity to increase the metabolic rate. Such a suggestion is in keeping with his view that many conditions are associated with an increased cellular utilization of cortical hormone and that epinephrine is

not of the adrenal cortical hormones. However, the close physiological relationship between the organs concerned allows deductions from the former to be applied to the latter.

Recently, the decline in either the blood lymphocytes or eosinophils has come into widespread use as a measure of adrenal cortical activation, or more properly as a measure of an increased blood level of adrenal cortical hormones. With this technique Godlowsky (1948), Recant *et al.*, (1950), and many others have shown that the subcutaneous or intravenous injection of epinephrine in many species, including man, is followed by a fall in the number of circulating eosinophils.

It has also been shown by Long and his colleagues that the capacity of epinephrine to influence either the level of adrenal cholesterol, ascorbic acid or number of circulating eosinophils can only be demonstrated in animals with an intact hypophysis.

Investigators in a number of laboratories, using a variety of species of animals, have established that epinephrine can bring about an increased rate of adrenal cortical secretion. It is also now agreed that this augmentation of adrenal cortical secretion follows a preliminary release of ACTH from the hypophysis and consequently the explanation for this effect of epinephrine is to be sought in the study of those mechanisms that control the secretion of ACTH. It is upon the nature of these mechanisms that disagreement still exists between the various laboratories engaged on this problem.

Conditions Associated with Augmentation of ACTH Secretion

Except possibly under exceptional or abnormal circumstances there is no increased secretion of adrenal cortical hormones without a prior release of ACTH from the anterior pituitary. The possible exceptions are the exposure of perfused adrenals to relatively high concentrations of histamine or potassium (Vogt, 1951), and the cyclic variations noted in hypophysectomized spayed rats in which artificial cycles were

subcutaneous injection of epinephrine does not prevent the usual eosinopenia. It would appear from these experiments that both histamine and epinephrine rapidly release ACTH and do so within a time which would appear to preclude this effect as being due to any great increase in the cellular utilization of cortical hormone.

The apparent rapidity of the response of the hypophysis is however in keeping with the time relationships which we have come to accept as characteristic of a neural or neuro-humoral mechanism. Furthermore, many stimuli that are associated with ACTH secretion are of a kind that would seem to preclude any metabolic disturbance as the immediate cause of its release.

The admission that there are circumstances associated with ACTH release that cannot be adequately accounted for by a decline in the blood level of adrenal cortical hormones does not exclude from further consideration the importance of this type of regulation. In my opinion it has been adequately proved that a purely humoral mechanism can regulate the secretion of ACTH.

Thus, the adrenal cortical hypertrophy and hyperplasia that follows unilateral adrenalectomy or adrenal demedullation would appear to be effected by humoral means alone. The hypertrophy of the adrenals of the intact partner following bilateral adrenalectomy in its parabiotic twin (Li and Pan, 1940) would also seem not to require the participation of a neural or neuro-humoral mechanism. The observation of Ingle, Higgins and Kendall (1938) that the injection of adrenal cortical steroids is followed by adrenal cortical atrophy is also evidence that the secretory rate of ACTH may be influenced by the blood level of adrenal cortical hormones. Finally, it has been shown both by ourselves and by Gordon (1950*a*) that traumatization of areas of the body deprived of their innervation is followed by increased adrenal cortical secretion. In our experiments, laparotomy with intestinal manipulation failed to cause eosinopenia in one hour in rats with a high spinal section but nevertheless still produced a marked fall

non-specific in this sense. This view is in keeping with the general hypothesis advanced by him that the regulation of both adrenal cortical and ACTH secretion is effected by the blood levels of these hormones. This regulation is of such a kind that a fall in the blood level of adrenal cortical hormones, as a result of increased cellular utilization, is in itself a stimulus to the secretion of ACTH by the hypophysis. Conversely, a rise in the blood level of cortical hormones decreases the rate of ACTH secretion. By direct evidence and by analogy with the relationship between the blood levels of the other trophic hormones and those of their target organs, there is much to give support to this hypothesis. Consequently if this is to be accepted as the sole mechanism concerned with the secretion of adrenal cortical hormones, the explanation for the effect of epinephrine on this secretion is to be found in what may be termed the purely metabolic effects of this hormone. Unfortunately there is still inadequate understanding of the nature of these metabolic effects of epinephrine, for as Griffith (1951) in his detailed account of this subject points out, the calorigenic (metabolic) effect cannot be considered as a unitary response but one that is an integration of several reactions including those of the vascular system.

(*b*) The chief difficulties in accepting a mechanism based solely on the reciprocal blood levels of adrenal cortical hormones and ACTH as the only regulator of adrenal cortical secretion are: first, the rapidity with which the pituitary-adrenal cortical system responds to suitable stimuli, and secondly the character of some of the stimuli that evoke a response.

Thus Gray and Munson (1951) have found that after the intravenous injection of histamine in the rat ACTH discharge occurs *in a matter of seconds. While it is true that the use of* intravenous histamine as a test of these regulatory mechanisms raises difficulties of its own, a similar rapidity of response has been observed in our laboratory to follow stimulation of sensory nerves, while Love (1950) has reported that adrenalectomy carried out in rats within ten minutes after the

subcutaneous injection of epinephrine does not prevent the usual eosinopenia. It would appear from these experiments that both histamine and epinephrine rapidly release ACTH and do so within a time which would appear to preclude this effect as being due to any great increase in the cellular utilization of cortical hormone.

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four hours after the injury. Adrenal demedullation did not prevent this delayed fall.

The point at issue at the present time would appear to be not whether there is both a rapid and a more slowly acting mechanism for the release of ACTH, but the nature of the more rapidly acting mechanism.

(c) I have pointed out that epinephrine which is released with great rapidity and under a variety of circumstances might well be the agent that is responsible for the rapid release of ACTH. As epinephrine release may be no more than a reinforcement of the nervous discharge and bear little or no relation to some specific neural or neuro-humoral mechanism of ACTH release, we have carried out a series of experiments designed to test (i) whether the removal of the adrenal medulla alters the secretory response of the anterior lobe; (ii) whether the direct application of epinephrine to anterior lobe tissue will cause ACTH secretion; and (iii) whether after exclusion of all possibility of reflex epinephrine discharge there still exists in the brain stem any mechanism that can cause a prompt release of ACTH.

We have found in our experiments after adrenal demedullation and the elapse of an interval of 4 to 7 weeks, that the immediate fall (1 hour) in eosinophils observed after exposure to cold, laparotomy, histamine or insulin injection is greatly diminished. The effect of insulin hypoglycæmia on adrenal cortical secretion is of some interest since it is well known that below a blood glucose level of 50 mg. per cent sympathetic excitation and epinephrine discharge regularly occur. Dury (1950) and Munroe (1951) have also found that adrenal demedullation is followed by a failure of adrenal cortical response to insulin hypoglycæmia, as judged by the absence of eosinopenia or a decline in adrenal ascorbic acid. On the other hand Gordon (1950*b*) has reported that insulin, exposure to cold and histamine injection still bring about a fall in the ascorbic acid content of the demedullated rat adrenal. This author does not submit any comparison of the responses of the adrenal demedullated with those of normal animals, so it

is not possible to say whether or not any alteration in the magnitude of response has occurred. In our laboratory we have encountered considerable difficulties in attempting to prepare adrenal demedullated rats in which the ascorbic acid content of the regenerated gland approached the values found in the intact organs. Furthermore, considerable variation in the control of the ascorbic acid in the regenerated glands has also been found.

In an attempt to determine whether the direct application of epinephrine to anterior lobe tissue is followed by ACTH release, we have carried out the following experiment. Rats were hypophysectomized and a piece of anterior lobe from a donor animal was implanted in the anterior chamber of one eye. After a sufficient period had been allowed for the growth of the transplant, the response of this tissue to the subcutaneous injection of epinephrine (20 micrograms), as well as to the painful stimulus associated with the subcutaneous injection of 1 ml. of 10 per cent NaCl was determined. In those animals in which an eosinopenia occurred, indicating a viable transplant, a very small quantity of epinephrine (0 2 micrograms) was injected into the anterior chamber of the eye bearing the transplant. In all seven animals used, a fall in eosinophils of equal magnitude to that produced by subcutaneous epinephrine was observed. Similar injections into the normal eye did not produce any fall in eosinophils. Finally the eye bearing the transplant was removed, after which no response to injected epinephrine was obtained. These experiments appear to indicate that anterior lobe tissue may be directly stimulated to secrete ACTH by small quantities of epinephrine. If these results are confirmed, many of the observations on the conditions surrounding ACTH secretion can be interpreted in terms of the release of epinephrine that is associated with them.

The third set of experiments I would like to present are concerned with an attempt to demonstrate whether or not a release of ACTH can occur in rats in which all possibility of reflex epinephrine discharge has been excluded by section of

the spinal cord at the level of the third thoracic vertebra some weeks previously.

If a few drops of 10 per cent NaCl are injected beneath the scalp of normal rats it produces a painful stimulus lasting a few minutes. It is followed by an eosinopenia that is evident one hour later and persists for at least four hours. However, similar injections under the scalp of spinal rats do not cause any *significant change in eosinophils either one or four* hours after the injection of the hypertonic salt solution, even though an equal degree of pain and discomfort is evident.

It would appear from these experiments that although the *sensory pathways from the skin and underlying tissues to the* thalamic and hypothalamic centres were activated in both the normal and spinal rats, it was only in the former where reflex secretion of epinephrine was possible that ACTH secretion followed such stimulation. In other words, this experiment suggests that there does not appear to be any mechanism within the cervical cord, brain stem, hypothalamus, or pituitary stalk which is capable of rapidly effecting a discharge of ACTH under conditions in which epinephrine secretion is abolished.

Consequently, we have tentatively suggested that a dual mechanism is responsible for ACTH secretion in the diverse and different circumstances under which it occurs. It would seem that we are observing the interplay of two connected mechanisms, one a self-regulating humoral system involving merely the relative blood concentrations of ACTH and adrenal cortical hormone, the other representing a part of the response of the autonomic nervous system. The activation of the autonomic nervous system with the release of its reinforcing hormone, epinephrine, is able to bring about with great rapidity the release of ACTH and hence of cortical hormone to meet the immediate requirements of the organism, while the purely humoral system comes more slowly into operation and may not be operative at all if the pressure on the organism is of short duration.

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NEURAL CONTROL OF SECRETION OF ACTH*

JOHN R. BROBECK

There now appears to be general agreement that the central nervous system or, more particularly, the hypothalamus is capable of stimulating the anterior pituitary gland to secrete adrenocorticotrophic hormone. Somewhat less well determined are the circumstances under which this stimulation occurs, while the mechanism, whether by a direct or an indirect activation, is the subject of no little debate. From the hypothesis proposed by Long and Fry (1945) that epinephrine may be an important stimulus to ACTH secretion, it might be inferred that the rôle of the central nervous system and the hypothalamus is expressed through their connections with the autonomic nervous system and the adrenal medulla. Both the tegmentum of the brain stem and the hypothalamus have been shown to be concerned with autonomic responses by the experiments of Karplus and Kreidl, Houssay, Cannon, Bard, Ranson, and their respective colleagues, as well as by many other workers.

Neural pathways which would be expected to take part in this activation may be summarized as follows, using cutaneous pain as an example of a stimulus which will eventually excite the adrenal cortex:—

(1) Conduction of afferent impulses over small myelinated and unmyelinated fibres through the dorsal roots to the spinal cord;

(2) Synapse in the substantia gelatinosa, with commissural neurones conducting to the lateral columns of the opposite side of the cord;

*Representing the studies of C. N. H. Long, Edith G. Fry, William V. McDermott, Ricardo R. Rodriguez and John R. Brobeck. From the Department of Physiological Chemistry, and the Laboratory of Physiology, Yale University School of Medicine, New Haven, Connecticut.

(3) Ascent through the lateral spino-thalamic tract to terminations in the thalamus, possibly within the ventral portion of the lateral nucleus;

(4) Connections, anatomically still obscure, with the hypothalamus—with neurones capable of exciting the sympathetic or thoracolumbar outflow;

(5) Descending connections from the hypothalamus, probably relayed through medullary autonomic mechanisms, ending finally within the intermediolateral cell column of the upper thoracic segments;

(6) Preganglionic neurones distributed through the white rami and splanchnic nerves to the adrenal medulla. From the medulla, epinephrine may be presumed to be carried to the anterior pituitary gland where ACTH is released, thus exciting the adrenal cortex to greater secretion.

All of these neural pathways have been well recognized for many years upon physiological evidence, and all, except the thalamo-hypothalamic connection, have been demonstrated anatomically. Their possible significance in regulating anterior pituitary function, however, had not been seriously investigated prior to the formulation of the hypothesis of Long and Fry.

In order to test their hypothesis further, experiments have been designed to interrupt at various levels the mechanism outlined above. Three types of preparation have been used, namely, rats with both adrenal medullæ removed, chronic spinal rats, and rats with lesions in the thalamus and hypothalamus Results of these experiments have been reported by McDermott and his associates (1950), whose conclusions may be summarized by saying that after interruption of this autonomic mechanism, activation of the adrenal cortex is delayed following exposure to cold, subcutaneous injection of epinephrine, or surgical operations. McDermott also concluded that there must be another mechanism, in addition to the autonomic-epinephrine complex, which can stimulate ACTH secretion, since in experiments of four hours' duration the cortex did increase its hormonal output (as measured by

blood eosinophil levels). The delays noted in one-hour samples in all three types of rats were taken to mean that the autonomic nervous system and adrenal medulla serve as a "trigger mechanism" bringing about a rapid excitation of the anterior pituitary, to be followed in most instances by the action of other, more slowly aroused influences which persist longer in their effects upon the hypophysis.

In McDermott's study the adrenal demedullated, spinal, and diencephalic animals were alike in their responses save in this one respect, that during cold exposure the spinal rats showed in four hours a much greater eosinopenia. The cold appeared to be more severe in its effect on those rats which, with the spinal cord severed at the third thoracic segment, could not fully bring into play the autonomic and somatic reactions of piloerection, vasomotor changes, postural adjustments, and shivering, all of which normally help to conserve heat or to increase heat production.

More recently, *Miss Edith* Fry and Dr. *Ricardo R.* Rodriguez, in Dr. Long's laboratory, have been repeating and extending these studies, and have been able to confirm many of the conclusions of McDermott *et al.* We now have in the combined series a total of twenty-five rats with lesions of the diencephalon, of which possibly seventeen showed delayed eosinopenia following cold exposure, laparotomy, or subcutaneously injected epinephrine (Fig. 1). The series is not large enough, nor are most of the lesions localized well enough, to allow us to decide just what region of the diencephalon must be destroyed to prevent the autonomic response. The smallest effective lesions which we have thus far produced have been of the type pictured in Fig. 2, involving the ventral portion of the anterior thalamus just dorsal and lateral to the para ventricular nuclei of the hypothalamus. In all of the animals with lesions like these the paraventricular nuclei were at least slightly injured, although it is questionable whether the injury was severe enough to have interfered with the function of these neurones. (This amount of damage to the supraoptic nuclei would give no sign of diabetes insipidus.) On the

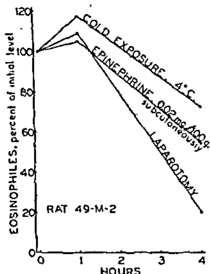


FIG. 1 Eosinopenia in a rat with bilateral lesions in the thalamus (Rat 49-M-2.)

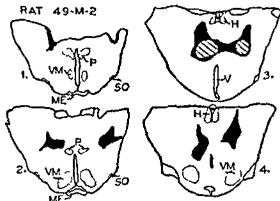


FIG. 2. Drawings from sections through the diencephalon of Rat 49-M-2. Lesions are solid black or cross lined, nuclei are outlined with dots

H Habenula.
ME Median eminence
O Nucleus ovoideus
OC Optic chiasm.
OT Optic tract.
P Paraventricular nucleus.
SO Supra-optic nucleus.
V Third ventricle
VM Ventromedial nucleus

other hand, this same degree of injury to the paraventricular nuclei and, in one or two cases even more extensive involvement, has been found in rats which showed normal adrenal cortical responses. It appears, therefore, that the paraventricular nuclei are not a part of the autonomic reflex responsible for ACTH secretion, but we cannot now identify the neurones which are. The fact that these smaller lesions are mainly thalamic in position has led us to consider whether they may be interrupting the thalamo-hypothalamic connection.

The series also includes a few rats with severe damage or even complete destruction of the paraventricular nuclei



FIG. 3 Drawings from sections through the diencephalon of Rat 49-M-23. Symbols are the same as in Fig 2

(Fig. 3). Their reactions were similar to those of rats with the smaller lesions described above. Of particular interest is the four-hour response of the rats with large lesions, since it shows the normal eosinopenia (Fig 4). This seems to indicate that the paraventricular nuclei are not required for the delayed, or so-called "metabolic" phase of the activation.

Miss Fry has also been able to confirm the essential part of McDermott's experiments where a painful stimulus was applied above the level of cord section in spinal rats. One ml. of 10 per cent saline injected subcutaneously produces a striking, but purely transitory, response in normal rats, which is accompanied by a 40 per cent fall in blood eosinophils within one hour. Spinal rats do not show this fall within the hour after saline is injected in the back of the neck where the

skin retains its normal innervation and central connections. In Miss Fry's studies only one out of six animals showed a significant eosinopenia in one hour, and upon re-testing this particular rat the earlier result could not be confirmed. From these data we conclude that the brain stem and hypothalamus do not contain a mechanism capable of increasing ACTH output in response to pain.

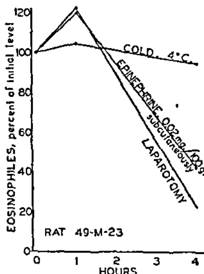


FIG. 4. Eosinopenia in a rat with large lesions of the anterior hypothalamus (Rat 49-M-23.)

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THE RÔLE OF ADRENALINE IN THE RESPONSE OF THE ADRENAL CORTEX TO STRESS OF VARIOUS KINDS, INCLUDING EMOTIONAL STRESS

MARTHE VOGT

DOCTORS Long and Brobeck have given us a beautiful account of their work on the rôle of adrenaline in controlling the output of ACTH in stress, and all that remains for me to do is to report on some experiments of my own, and in order to stimulate discussion, not to dwell too long on the many points of complete agreement, but to emphasize some minor points about which our experiments do not lead to the same conclusions.

Most authors (Gellhorn and Frank, 1949, Gershberg *et al.* 1950, Gordon, 1950*a*; Recant *et al.*, 1950; Vogt, 1947, 1951*a*) are now agreed that the response of the anterior pituitary to stresses like low and high environmental temperature, [illegible] not abolished [illegible] The question [illegible] n the adrenal medulla is unable to function. One fact renders the answer to that question difficult Consider the first five of those conditions of stress In all of them the release of adrenaline greatly helps the animal in withstanding the damage done by the stress, and the same condition, let us say the same dose of insulin, is a much more severe stress for the animal in which the medulla has been removed than for the normal control Therefore, if a response of the pituitary in the operated animal is equal to that of the normal, it does not represent an equal response to the same, but to a severer degree of stress. A condition in which this difficulty does not arise is the injection of β-tetrahydro-naphthylamine (β-Tetra). This compound,

which stimulates both adrenal medulla and central sympathetic centres, is tolerated by the animal with demedullated adrenals equally well as or even slightly better than by the normal control. Nevertheless, as shown in the first figure, the depletion in the adrenal ascorbic acid of the rats subjected to adrenal demedullation is only slightly less than that of the normal controls. This difference appears even greater than it really is, because, in the demedullated rats, the initial ascorbic

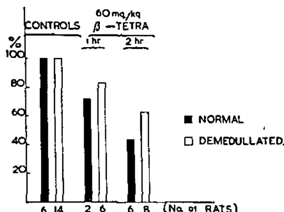


FIG 1 Ascorbic acid in rat adrenals, percentage of controls Effect of subcutaneous injection of β-tetrahydro-naphthylamine.

acid concentration before stress is applied is lower and the possibilities of depletion are therefore more limited.

If, then, the size of the response is not greatly altered by demedullation, is the speed reduced? Dr. Long has emphasized the slowness of what he has termed the "metabolic" phase of the response to stress. Now the ascorbic acid falls only slowly under the effect of β-Tetra, as seen in the small response one hour after the operation, but it does so in normal and operated rats alike, because the metabolic effects of β-Tetra develop slowly Would the same hold if the metabolic response were rapid? Here is a point where my experiments

led me to conclusions which differ somewhat from Dr. Long's. A number of authors (Gordon, 1950*a*; Gray and Munson, 1951) have seen depletion of adrenal ascorbic acid in demedullated rats or in rats given large doses of tetraethylammonium chloride as early as 1 hour after the beginning of stress. As shown in Table I, the process can be much faster still: a significant fall of ascorbic acid to 72 per cent of the control value was caused in demedullated rats in the course of 10-12

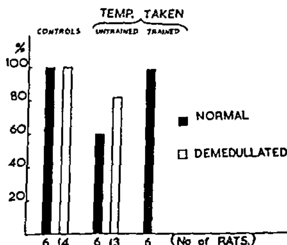


FIG 2. Ascorbic acid in rat adrenals, percentage of controls
Effect of "emotion" (measurement of rectal temperature)

[illegible] by performing an operation under ether. The operation [illegible]

nerves.

The second point I wish to raise is that of the response of the demedullated adrenal to emotional stress (Vogt, 1951*b*). My own conclusions are not the same as those of Gershberg *et al.*, but I think I know the cause for this difference. First, I shall show you the results (Fig. 2). Groups of rats, normal and demedullated, had their rectal temperature taken with a small

thermometer three times during the course of 20 minutes and were killed an hour later. Provided the rats had not previously experienced such measurements of temperature, their adrenal ascorbic acid fell by 40 per cent in the normal and by 17 per cent in the operated rats. Small though it is, this latter fall is statistically significant, and constitutes an emotional release of ACTH caused by an unfamiliar procedure. Adrenocortical responses of demedullated rats to sensory stimuli have also been reported by Gordon (1950*b*), who demonstrated that in such animals traumatizing of an innervated leg caused a greater adrenal response than injury to a denervated leg. Ronzoni and Reichlin (1950) believe that the lack of correlation between fall in adrenal ascorbic acid and rise in blood sugar seen on stimulation of the central end of the sciatic nerve shows that the response of the adrenal cortex to sensory stimuli is not dependent solely on the release of adrenaline.

How are we to account for the discrepancy between these results and the two sets of experiments reported from Yale? I think it is all a matter of thresholds. In the experiments by Gershberg *et al.*, in which ascorbic acid depletion was used as an index of release of ACTH, the initial ascorbic acid concentration in the glands of the control animals was too low for a very small effect to show. It was fortunate that the control rats in my own experiments had high ascorbic acid concentrations and so rendered conditions more favourable for the detection of small depletions. In the results of McDermott *et al*, counts of the circulating eosinophils were used in order to follow, in spinal rats, the effect of injecting hypertonic saline on the release of ACTH. I hope Dr. Long would agree that depletion of ascorbic acid in the rat is obtained with much lower doses of ACTH than is a similar percentage depression of eosinophils. It would then not be surprising if the small effect of emotion which persists after adrenal demedullation is not reflected in a depression of the blood eosinophils.

How can one explain this effect of emotion on the demedullated adrenal?

The first possibility is that, in the absence of the adrenal medulla, enough adrenaline or sympathin may enter the general circulation during emotion to account for a release of ACTH. Sympathin contains a high percentage of *nor*adrenaline. *Nor*adrenaline can easily be excluded as a possible cause for this phenomenon, since it has been shown by many authors (first by Nasmyth, 1949) that it releases ACTH only when acting in high concentrations. The possibility that *extra-medullary adrenaline* may be responsible has been tested directly in the following way:—

The plasma adrenaline of rats was estimated under four different conditions:—

(1) Normal rats, anæsthetized with ether, were subjected to cannulation of one carotid artery, injection of heparin and collection of blood from the carotid while the central end of the brachial nerves was stimulated electrically.

(2) Demedullated rats, treated like group 1.

(3) Demedullated rats, injected i.m. four times with 1 μg. adrenaline per 100 g. rat at intervals of 15 minutes. Blood was obtained from the carotid 6–8 minutes after the last injection.

(4) Demedullated rats, treated as under (3), the dose of adrenaline being 2 μg. instead of 1 μg.

The results, together with adrenal ascorbic acid estimations done on the same animals, are shown in Table I.

Blood adrenaline estimations were carried out by making extracts of plasma with acid alcohol, subjecting them, after purification, to paper chromatography, and testing the eluates from the paper by their power to inhibit contractions of the rat's uterus elicited by carbaminoylcholine (Gaddum, Peart and Vogt, 1949; Crawford and Outschoorn, 1951).

Stimulation of afferent nerves produced adrenaline concen- [illegible]

of 12 similarly treated demedullated rats. I.m. injection, [illegible] demedullated rats, of doses of adrenaline which just caused a detectable depletion of ascorbic acid produced concentrations of blood adrenaline which were detectable by the assay.

The inference is that circulating extramedullary adrenaline is not responsible for the release of ACTH in emotional stress of the demedullated rat.

The alternative is to assume that the effect is produced by direct stimulation of hypothalamic centres which, according to de Groot and Harris (1950), are responsible for a release of ACTH in the rabbit subjected to emotional stress. It is not unreasonable to assume that similar centres exist in the rat.

Table I

PLASMA ADRENALINE AND ADRENAL ASCORBIC ACID IN RATS SUBJECTED TO VARIOUS TREATMENTS

Treatment	*Plasma adrenaline mμg /ml*		*Adrenal ascorbic acid (percentage of controls) demedullated*	*Interval mins.*
	Normals	*Demedullated*		
Operation (ether)	2 3 (5 rats)	<0 5 (12 rats)	72 (10 rats)	12
4×1 μg /100 g Adrenaline I m.	—	0 2 (1 rat) 0 5 (1 rat)	83 (2 rats)	60
4×2 μg /100 g. Adrenaline I m	—	1 0 (1 rat) 2 0 (1 rat)	58 (2 rats)	60

The table also demonstrates the fact mentioned earlier, that, in the demedullated animal, a significant depletion of adrenal ascorbic acid can occur within 12 minutes from the beginning of a stressing procedure, provided the physiological consequences of the stress take place sufficiently rapidly.

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DISCUSSION

PRUNTY: I wonder how far these excellent experiments of Dr. Long and his colleagues can be applied to the human? I'm both seeking information and offering it. We have found that doing four-hour eosinophil responses to adrenaline injected subcutaneously in humans is a very chancy sort of business and doesn't seem to obey any rules. That has been borne out I think by the observations of Broch and ... Some

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to the morning dose of ACTH on a six-hour régime is 100 per cent, there is always a significant and reasonable increase in the excretion rate, by our technique about 3 mg per day. In a patient with rheumatoid arthritis whom we treated with multiple doses of adrenaline over a period of nine days with doses up to 4 5 mg., divided into six and administered subcutaneously, there was a complete disappearance of

produced

HUME: There are a fe

4-hour fall by accepting Dr. Sayers' theory that trauma to tissue decreases the circulating corticoids in the blood stream and that this decreased level produces ACTH release. I don't know whether Dr Long

still believes that or not. We feel that operations done on the legs of animals with high spinal section in which there is no release of adrenal corticoids, and the speed of ACTH release following trauma in the [illegible]

some other substance.

And the third and most important difference is where epinephrine [illegible]

animal, so that it doesn't act by means of releasing epinephrine.

With regard to the effectiveness of epinephrine in producing an eosinophil fall, there are widely different responses to this substance in different species. In fact, if one takes dogs alone, one finds that in different breeds of dogs there are different responses to epinephrine. For instance, we have tried injecting coach dogs with the same dose of epinephrine which produces a good fall in other dogs, and found that 5 out of the 6 had no fall at all with epinephrine and the sixth had a [illegible]

differences between dogs and rats.

Dr. Thorn and his co-workers appear to have demonstrated a very curious phenomenon, which he hasn't reported yet. Twelve total bilateral adrenalectomies have been done in humans, and in those people who have been given epinephrine tests post-operatively several have shown a perfectly normal eosinophil fall in response to epinephrine, in the absence of both adrenals. (This does not occur in the dog.) [illegible]

[illegible]

talk that we've had, by saying that the dose of epinephrine which is put in the eye with the graft is a much smaller dose than would give an effect if injected in the periphery of the animal. I don't know at what point in t[illegible]
end of the [illegible]
from the [illegible]
going from the eye to the hypothalamus.

Secondly, both epinephrine and histamine, when placed directly on tissue, are very damaging agents We had to give up the use of subcutaneous [illegible]
slou, [illegible]
epin [illegible]
dam [illegible]
I [illegible]
and [illegible]
location of the lesions which we found were most effective. I also feel that the injection of 10 per cent sodium chloride under the skin is a much less traumatic stimulus than operative trauma

Long: I would like to repeat something I said this morning, that failure to respond in eosinopenia to epinephrine is one thing, and these experiments of Thorn that Dr. Hume has quoted on the fall of eosinophils in the adrenalectomized animal, another. Nevertheless, Dr. Vogt has very clearly shown that epinephrine does produce an increased adrenal cortical secretion. I think we are getting a little confused by

Dr. Hume asked, why did we pick on epinephrine, why don't we pick on histamine or acetylcholine, or blood glucose or potassium? The answer is that epinephrine, in amounts which we know can occur in the blood under these circumstances, is a stimulus to ACTH secretion, while the quantities of histamine and of acetylcholine that are used to produce comparable falls in eosinophils are not normally encountered in the blood. After stimulation of a sensory nerve, for example, one does not have in the blood quantities of acetylcholine or histamine that are comparable to the known increases in epinephrine, and these blood levels of epinephrine, as Dr Vogt has just shown, are perfectly capable of stimulating the secretion of the adrenal cortex.

[illegible]

purely humoral control of the adrenal cortical secretion. The experiments that Professor Houssay mentioned yesterday on parabiosis are very difficult to explain unless you assume that there is some type of

a too rigid separation between the two types of response, or two types of secretion of ACTH. One is not necessarily followed by the other. They may occur almost simultaneously under some circumstances; under other circumstances one may be the predominant mechanism, and, as I mentioned, the other may not appear at all, or only appear to a small degree. It depends on the circumstances.

VOGT. I would like to mention an observation made on the eosinophils,

responses are unspecific and one has to be very careful when the conditions of the experiment are complex.

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op
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this case in the rat?

with histamine

HARRIS: The second point was with regard to Sayers's view. I am

talk that we've had, by saying that the dose of epinephrine which is put in the eye with the graft is a much smaller dose than would give an effect if injected in the periphery of the animal. I don't know at what point in the periphery of the animal this is injected, but if it is in the

and Dr. Brobeck have described were completely different from the location of the lesions which we found were most effective. I also feel that the injection of 10 per cent sodium chloride under the skin is a much less traumatic stimulus than operative trauma.

LONG I would like to repeat something I said this morning, that failure to respond in eosinopenia to epinephrine is one thing, and these experiments of Thorn that Dr. Hume has quoted on the fall of eosinophils in the adrenalectomized animal, another. Nevertheless, Dr. Vogt has very clearly shown that epinephrine does produce an increased adrenal cortical secretion I think we are getting a little confused by our reliance on the change in the eosinophils as the decisive measure of

Dr. Hume asked, why did we pick on epinephrine, why don't we pick on histamine or acetylcholine, or blood glucose or potassium? The answer is that epinephrine, in amounts which we know can occur in the blood under these circumstances, is a stimulus to ACTH secretion, while the quantities of histamine and of acetylcholine that are used to produce comparable falls in eosinophils are not normally encountered

into a normal rat there is a rapid fall of the eosinophils within 30 minutes or an hour; but in an animal in hypoxia there is an *increase* of eosinophils for an hour or more, in spite of the fact that there is immediate release of adrenaline in hypoxia; and after this time there is a definite decrease. In both cases there is an increase of secretion of adrenaline. There is something other than adrenaline acting, because if adrenaline alone were acting, you would get only a rapid fall in the eosinophils.

BROBECK: We have observations like Professor Houssay's in some respects, and we've been giving cortical extract to demedullated animals because preliminary experiments suggest that by this procedure *the ascorbic acid in the demedullated glands may be restored to the* normal level. Miss Fry has some experiments that tend to show that in [illegible]

the spinal animal, for example, is to a certain extent fortuitous, because in that period the count has first risen and then begun to fall.

LONG: I have mentioned several times some of the difficulties encountered in using the level of blood eosinophils as a criterion of adrenal cortical secretion. I would like to point out that the contraction of the spleen that occurs in many circumstances associated with ACTH secretion may actually cause a rise in the eosinophils, at least for a [illegible]

interesting to mention some experiments performed recently in our laboratory and published in Nature (Selye, H., 1951, *Nature, Lond.*, 168, 149). In these experiments, Dr. Selye showed that ACTH can synergize the thymolytic effect of a corticoid hormone such as cortisone, even in the absence of the adrenal cortex. While these results were obtained by studying the lymphatic tissue and not the eosinophils, they can, however, be connected with those of Thorn and Forsham on the eosinophil fall in adrenalectomized humans. Both these observations suggest that a peripheral action of ACTH may be responsible for the eosinophil fall and for the thymolytic action.

In connection with the ascorbic acid depletion of the adrenal after the administration of several drugs, I would like to mention the work of some of my colleagues (Dordoni, F., and Fortier, C., 1950. *Proc. Soc exp. Biol. Med*,. 75, 815). In this work the adrenal ascorbic acid response to independent and simultaneous administration of eserine and atropine was studied in the normal and hypophysectomized rat.

very sorry Dr. Sayers is not here today, for we could then ask him personally. But it seems to me very difficult to explain the adrenal hypertrophy you get with chronic stress on Sayers's hypothesis According to Sayers the output of ACTH is stimulated by a fall in the level of the blood corticoids. However, the adrenal hypertrophy that follows prolonged stress implies a raised blood level of adrenal cortical hormone coincident with a raised blood level of ACTH Some other factor must come into the picture, at least under these conditions

LONG: I also regret very much that Dr. Sayers is not here to defend his reciprocal blood level hypothesis. I agree with Dr. Harris that there are difficulties when one comes to these chronic experiments. I would also like to point out that it's well known in man that after burns or fractures the increase in adrenal cortical steroids in the urine becomes very marked, and I don't see how that is compatible with a theory which supposes that the blood level of the adrenal cortical steroids at that time is reduced.

HARRIS: With regard to these anterior pituitary grafts. Grafts in the eye, I think I'm right in saying, do not maintain adrenal glands of normal weight. In Dr. Sayers's experiments his grafted animals had adrenals that were as atrophic, in many cases, as those in the hypophysectomized controls, and I think that most workers have found that grafts in the anterior chamber of the eye will result in adrenal atrophy to greater or less extent. Grafts under the median eminence, however,

atrophied, presumably the physiological requirements for increased adrenal cortical output are not sufficient to stimulate the secretion of

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PRESUMPTIVE HYPOTHALAMIC CONTROL OF SPONTANEOUS OVULATION

JOHN W. EVERETT

REFLEXOGENIC activation of the rabbit adenohypophysis leading to ovulation is apparently a rapid process which is completed within about one minute after coitus. The final link in the reflex is thought to involve an adrenergic neurohumor (Markee *et al.*, 1948; Sawyer, Markee and Everett, 1950*b*) which is brought to the gland from the median eminence by the hypophyseal portal veins (Harris, 1948). The reflex can be blocked by the anti-adrenergic agents, Dibenamine or its congener SKF-501, when intravenous injection is completed within a minute after coitus (Sawyer, Markee *et al.*, 1947, 1949, 1950*a*). A cholinergic mechanism preceding the adrenergic one is assumed from the finding that intravenous injection of atropine will also usually prevent ovulation when injection begins within 15 seconds and ends within 30 seconds post coitum. This finding was recently supported by very similar results with another anticholinergic drug, Banthine (Sawyer, Markee and Everett, 1951). Central components of the reflex evidently operate so rapidly that no interference with ovulation follows the induction of profound narcosis with pentobarbital or pentothal within 15 to 30 seconds post coitum.

Spontaneous ovulation in the rat is equally dependent, we think, on a chronologically limited neurohumoral stimulus to the hypophysis, qualitatively similar to that in the rabbit. Everett, Sawyer and Markee (1949) demonstrated that in cyclic rats ovulation can be prevented either by intravenous injection of Dibenamine or by subcutaneous injection of atropine before 2 p.m. on the day of pro-œstrus (4-day cycle). Similar treatment at 4 p.m. does not usually interfere with

Eserine as well as atropine induced a marked decrease of ascorbic acid which was enhanced by the simultaneous administration of the two drugs. The fall of ascorbic acid was prevented by hypophysectomy. These results indicate that two substances pharmacologically opposite may have the same action on adrenal ascorbic acid. I think that we could ascribe these results to the "non-specific action" of the drugs.

QUERIDO: I just want to make a remark about the relation between the thyroid and the adrenals which might be important in the comparison of animals with eye grafts and animals which are not grafted. In thyroidectomized animals after about three months we find a significant decrease in the adrenal weight, and I wondered whether the animals with grafts in the anterior chamber of the eye shouldn't be given a preventive dose of thyroxine to keep adrenal function at normal level. This perhaps should come into consideration when considering different magnitudes of reactions.

LONG: It is an interesting suggestion that the metabolic rate might be low in these animals. As far as I know, nobody has measured the metabolic rate of animals that have an anterior pituitary graft in the eye.

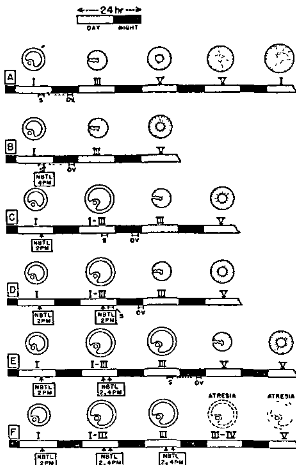


FIG. 1 Schematic representations of the normal 4-day cycle (A) and of the characteristic results of different régimes of pentobarbital treatment (B-F) Stages of the vaginal smears are indicated by Roman numerals above each time scale, while symbols above these show the corresponding follicle and corpus

ovulation. Identical results, with similar time relation have been obtained with intravenous SKF-501 (Sa Markee and Everett, 1950*a*), subcutaneous Banthine published) and the barbiturates (Everett and Sawyer, 1 pentobarbital in particular.

Pentobarbital Experiments

Studies with pentobarbital have shown that this agen *regularly prevent ovulation, when injection is properly* t and, in addition, they have given evidence that the ce (presumably hypothalamic) component of the "LH-re mechanism" has a 24-hour periodicity. Several of the ty experiments are summarized in Fig. 1.

Events in the normal 4-day cycle are represented in Fig On the day of pro-œstrus the critical hours for pitu activation (S) are shown to be between 2 and 4 p.m. O tion (OV) occurs between 1 and 3 a.m. next morning; l fresh corpora lutea are present at autopsy later that Injection of pentobarbital (or any of the other blocking ag at 4 p.m. usually does not modify these results (Fig. However, as shown in Fig. 1*c*, injection of pentobarbit 2 *p.m. during pro-œstrus delays events exactly* 24 *h* Stimulation of the hypophysis occurs on the second between 2 and 4 p.m. and ovulation follows that night. stimulus may be blocked again on the second day (Fig. 1*e* pentobarbital at 2 p.m. if a supplementary injection is g 1½ to 2 hours later The cycle is retarded another 24 ho ovulation now takes place during the third night. Block for 3 days in sequence (Fig. 1*f*) characteristically results in completion of a follicular cycle, and all of the large folli become atretic *on the fourth morning. At that time* vaginal smear is usually di-œstrous. New follicles are grow meanwhile and, in the absence of further treatment, p œstrus of a new cycle occurs about day 6 and the new folli ovulate that night.

Save for the fact that follicle generations are shorter-li and less overlapped, these experimental follicular cycles

appearance. This depletion of interstitial lipid appears to be a function of an excess of gonadotrophin, as suggested by the work of Claesson and Hillarp (1947). When ovulation has occurred, the tubal ova are easily visualized during the follow- [illegible] The ova are clearly seen through the transparent wall of the dilated segment. In the absence of ovulation this pronounced dilatation is not found.

Histologically the follicles of a fully blocked rat on the day after injection show no evidence of preovulatory swelling or luteinization of the granulosa (Fig. 3). The ovocyte nuclear membrane is intact. Secretion of secondary liquor folliculi is not apparent. The granulosa remains compact with its basal layer everywhere clearly separated from the theca interna by the basement membrane. The follicles have simply continued to grow. They continue to secrete œstrogen, as indicated by the prolongation of vaginal cornification into the third day (if, for example, a pentobarbital-blocked rat is allowed to survive that long—Everett and Sawyer, 1950). The blocking agent does not, then, greatly impair secretion of either FSH or whatever small amount of LH is necessary for the elaboration of œstrogen. It does prevent, selectively, the release of the additional amount of gonadotrophin which would cause preovulatory swelling, follicle rupture and luteinization.

Estimated Duration of the Ovulatory Stimulus in Rats

With the intention of learning more about the actual duration of the ovulatory stimulus in this species, we recently explored the interval between 2 and 4 p.m. during pro-œstrus (Everett, 1951). Forty-nine 4-day cyclic rats, in groups of 9 or 10 each, were given the standard blocking dose of atropine (cf. Sawyer, Everett and Markee, 1949) at one or another of the various times shown in Fig. 4 between 2.40 and 3.30 p.m. All of the rats were autopsied about 18 hours after injection.

rats appear to be analogous with those in the œstrous rabbit. The mechanism which is selectively blocked out is comparable, in certain respects, with the coital reflex in the rabbit. Both can be blocked by the same agents. Furthermore, of the extensive list of agents which will not block in the rabbit, several have been tried in the rat with equally negative results These are, specifically: the imidazoline adrenolytics; the non-adrenolytic derivative of Dibenamine, 2-dibenzylamino-ethanol; and the ganglionic blocking agent, tetraethyl-ammonium (Sawyer, Markee and Everett, 1950).

We have, therefore, formulated the hypothesis that spontaneous ovulation and reflex-induced ovulation are both governed by a comparable hypothalamico-hypophyseal linkage. In rats this apparatus is considered to be activated by a *hypothalamic centre in which resides the property of spontaneity* and which undergoes a well-defined diurnal fluctuation of excitability. Correlated studies have indicated that this excitability also depends importantly on œstrogen and progesterone levels (Sawyer, Everett and Markee, 1949; Everett and Sawyer, 1949; Everett, 1951).

The Ovary as an Indicator of Blockade

The effect of blockade of the LH-release mechanism, as registered in the ovary by the following morning, is well illustrated by the next two slides. (Two Kodachrome slides were shown, photographs of freshly excised ovaries from a Dibenamine-blocked rat and an untreated control rat, respectively. Both had been running parallel 4-day cycles. The first received Dibenamine at 9 a.m. during pro-œstrus and both were autopsied at the same hour of the next morning.) The first is characteristic of full blockade with any of the agents mentioned. The follicles are uniformly large and clear. The interstitial tissue remains markedly fatty as it normally is during pro-œstrus. In the absence of blockade (control ovary) there are full sets of fresh corpora lutea. The interstitial tissue is markedly depleted of fat, taking on a rather dull, watery



[illegible] segment of



FIG 3 Typical graafian follicle at about 10 a m of the morning after pentobarbital blockade (injection at 2 p m during pro- [illegible]

The 2 p.m. and 4 p.m. data (64 additional rats) included in the chart are taken from previously cited studies with both atropine and pentobarbital. It is apparent that in the majority of cases activation of the hypophysis began during the first hour and that it was often complete within that time. In fact, in 3 rats it was complete by the time the 2.40 injections took effect.

Among the 49 rats of the 2.40 to 3.30 groups, 9 (18·4 per

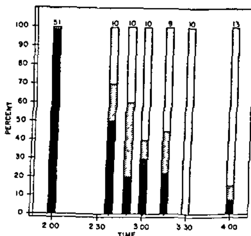


FIG. 4 Results of attempted blockade at various times during the critical 2 to 4 p m interval on the afternoon of pro-œstrus (4-day cycle) BLACK—complete blockade. SHADED—partial blockade WHITE—full ovulation. Number above each bar denotes the number of rats in that respective time-group

cent) were partially blocked, i.e. showing on the next morning intermediate effects between complete blockade and full ovulation. The minimal effect of LH release that was recognized was the presence of localized lutein patches (Figs. 5 and 6) in the walls of 2 or more follicles. The maximal effect short of full stimulation was the presence, in one pair of ovaries, of 5 very freshly ruptured follicles (cf. Fig. 8) and 7 follicles in late swelling (cf. Fig. 7). In several cases the serial sections disclosed 1 or 2 follicles in swelling, coexisting with others

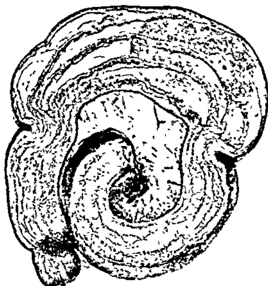


Fig. 2 Tubal ova as normally found in the dilated segment of the ampulla on the morning after ovulation Excised loop of ampulla compressed in physiological saline between slide and cover slip

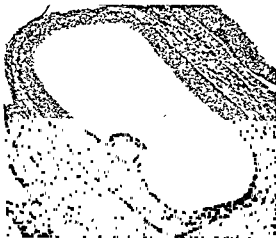


Fig 3. Typical graafian follicle at about 10 a m of the morning after pentobarbital blockade (injection at 2 p m during pro-œstrus) (*By permission of the editor of Endocrinology*)

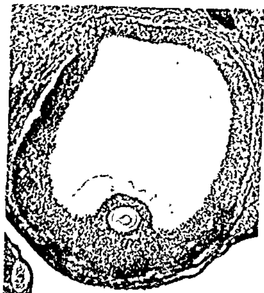


Fig. 5. Localized lutein plaque (upper left) in the wall of a follicle otherwise unaffected by luteinizing hormone.



Fig. 6. A lutein plaque similar to that in Fig. 5, under higher magnification. Theca and granulosa fused. Unaffected



Fig 7 Late preovulatory swelling, impending rupture Note polar body *Incomplete blockade by Dibenamine (cf Everett, Sawyer and Markee, 1949).*



Fig 8 Very recently ruptured follicle from same ovary as Fig 7

which were unaffected or which at most contained localized lutein patches.

While these intermediate effects undoubtedly represent release of less than the usual amount of luteinizing hormone, it is unlikely that this resulted from direct interference with the actual discharge of the hormone into the blood stream. Certainly none of the blocking agents has such an effect in rabbits; a species difference in this respect appears unlikely. It follows, therefore, that diminished release of luteinizing hormone resulted from interruption of hypophyseal activation *per se*.

Since the chance of interrupting a progressive process is a function of its length, it is possible to translate the percentage partially blocked into duration of stimulus. Calculations made on this basis have led to a rough estimate of 20 to 35 minutes. An extreme upper limit of 40 minutes is given directly by the 2.40 p.m. data, where 3 rats were fully activated. The lower limit of expectation ($P=0\cdot005$) calculated from Fisher and Yates' Table $VIII_1$ (1949) is 10 minutes. We have concluded that in the rat the spontaneous stimulus is far longer than the momentary reflex stimulus in the rabbit.

Reflex Induction of Ovulation in the Rat

Recognizing the high predictability of spontaneous pituitary activation in our rat colony and the selectivity of pentobarbital blockade on successive days, various procedures may be attempted to invoke activation "out of hours," so to speak.

There is in the literature some evidence of copulation-induced ovulation in this species under special conditions. Dempsey and Searles (1943) reported that after production of persistent œstrus by continuous light, copulation caused corpus luteum formation. Browman (personal communication) has stated that females exposed to continuous light will become pregnant. Rats which have spontaneously developed persistent œstrus will occasionally form active corpora lutea after mating (Everett, 1939, 1940), although a delay of several days often intervenes. We have been especially interested

recently in the question of whether ovulation can be induced by copulation outside the known critical hours for the spontaneous stimulus. Consequently the following experiment was carried out (unpublished).

The basic procedure was barbiturate blockade at the critical time during pro-œstrus and the following day (cf. Fig 1e). In about half the cases such animals came into heat during the intervening night and copulated when caged with known fertile males. The males were removed about 8 a.m. next day and the vaginas were examined for plugs and sperm.

In 9/55 trials this procedure resulted in ovulation which was directly referable to the copulation. In one animal, autopsied on day 3 (2 days after pro-œstrus), a set of well-organized corpora lutea was found, nearly as well developed as they would have been had no blocking agent been given. Cleaving ova were found in the fallopian tubes. In the other 8 rats pregnancy was established. By such means as back-dating from the placental sign, ovulation was assigned in each case to the first 24 hours of the experiment.

In the great majority of the remaining trials, where ovulation and pregnancy were not induced, the result was unexpectedly bizarre (Fig. 9). Follicular atresia was frequent in rats autopsied on the fourth morning and the incidence of fresh corpora lutea or luteinized follicles was relatively rare (contrast with Fig. 1e). During the succeeding cycle, however, fresh corpora lutea were regularly formed. Oddly enough, when animals were allowed to survive, these corpora lutea became activated and pseudopregnancy followed· a curiously delayed effect of the copulation 5 or 6 days earlier.

While in the past it has been customary to consider as analogous the coital reflex for ovulation in the rabbit and the induction of pseudopregnancy in rats, this appears to be erroneous. The analogy should be restricted to induction of ovulation in either species. Induction of pseudopregnancy does not appear to be a rapid reflex: witness the delayed response just mentioned and the earlier report by Greep and Hisaw (1938) that pseudopregnancy may be induced by

electrical stimulation of the cervix during the *preceding* diœstrous interval. I find it difficult to accept the view that a brief (reflex) stimulus to the hypophysis will set up in the gland itself a new pattern of secretion and maintain it for 10 to 14 days. Admittedly, the results of one of the stalk-sectioning experiments by Westman and Jacobsohn (1938) and the data of Taubenhaus and Soskin (1941) seem to favour

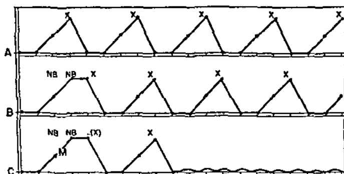


FIG. 9.

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B. Blockade with pentobarbital on the day of pro-œstrus and the following day (cf. Fig. 1, E) Ovulation during the third night.

C. Same basic procedure as B, but with copulation during the first night Corpora lutea formed during the second cycle become functional The undulating line represents pseudopregnancy.

the single stimulus. Both experiments, however, are open to reinterpretation in view of present-day knowledge.

Summary

The evidence outlined indicates that in female rats different mechanisms control hypophyseal secretion leading respectively to: (I) follicle growth and œstrogen secretion; (II) preovulatory swelling, ovulation and luteinization; and (III) activation of organized corpora lutea.

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DISCUSSION

Timiras. I would like to ask Dr. Everett what is his interpretation of the action of Dibenamine on LH release. Dr. Nickerson has already recalled that, although the adrenergic blocking action of Dibenamine usually overshadows all other actions of this compound, it must be kept in mind that no known drug is absolutely specific. Thus, observing that the injections of Dibenamine in the experiments of Everett, Sawyer and Markee were regularly followed by signs of extreme central nervous system stimulation, he suggested that this drug can inhibit LH release by a central nervous effect independent of adrenolytic [illegible]

[illegible]

œstradiol benzoate and were exposed to various types of stress One group received two injections of 0 9 mg of adrenaline each, another group two injections of 0 09 mg. of eserine, another group was injected with a 4 per cent solution of formalin, and in the last group the spinal cord was resected Twenty-four hours after exposure to stress all animals were sacrificed In all animals exposed to stress an increase in adrenal weight and a decrease in lymphatic tissue weight were found. Similarly, in about 40 per cent of the stressed rats we observed a blockade of LH release as shown by the loss of cholesterol-staining material in the ovary.

Everett: Your basic procedure, as I understand it, was the injection of œstrogen during early pregnancy, with examination of the corpora lutea two days later for cholesterol storage In the animals subjected to stress was there any interference with the induction of ovulation by œstrogen?

Timiras: The number of ova was also decreased in all stressed groups Dr. Dordoni and I wondered how our results would fit in with your idea of an adrenergic stimulation since drugs other than Dibenamine, such as adrenaline and eserine, and even formalin and spinal cord resection could preclude LH release.

Everett. We have used other substances than Dibenamine. In addition to Dibenamine, SKF 501, atropine and Banthine, we have used tetraethylammonium and the imidazoline adrenolytics, in amounts which one would expect to be inducive of stress. Under the conditions in which we administered these other agents there was no interference

Wh... is t... ver... ...is still to be defined.

Mechanism II, however, has been explored sufficiently for one to characterize it tentatively as similar to that in rabbits, save for its spontaneity. There is evidence here of neurohumoral control by way of the hypophyseal portal veins. While this neurohumoral apparatus may under some circumstances be activated reflexly in rats as in rabbits, it is usually activated in the former by a rhythmic centre presumably in the hypothalamus. Indications are that this centre undergoes diurnal variation in sensitivity and that it is, furthermore, potentiated by elevated œstrogen and progesterone levels.

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EVERETT: We have used other substances than Dibenamine. In addition to Dibenamine, SKF 501, atropine and Banthine, we have used tetraethylammonium and the imidazoline adrenolytics, in amounts which one would expect to be inducive of stress. Under the conditions in which we administered these other agents there was no interference

with the spontaneous ovulation mechanism. We did not try them in the pregnancy experiment.

TIMIRAS: We have found in our experiments that there was some relation between the weight of the adrenals and of the thymus and LH blockade. We believe therefore that inhibition of LH secretion from the hypophysis might be explained—at least in part—by a shift in the secretion of the pituitary.

EVERETT: That idea occurred to us. For that reason we not only took into account the injection of these other agents, which were not

EVERETT: No

SAWYER: In the rabbit dibenzylaminoethanol has an even more shocking effect on the animal than has Dibenamine, and yet the animals ovulated normally

TIMIRAS: I have been speaking of experiments in the rat, not in the rabbit.

SAWYER: The rat is, of course, a more difficult animal with which to work on the mechanism of Dibenamine action because of the long timing [illegible]
with [illegible]
used t[illegible]
stimu[illegible]
agents [illegible]

rather dangerous, that there may be local damage. I would like to know what Dr. Harris thinks about that.

HARRIS: I think that possibility that you are damaging at least some cells of the anterior pituitary does mean that you require fairly rigid controls.

MARKEE: I think we supplied the controls when we applied the acetylcholine.

SAWYER: And the KCN, and the electrodes.

HARRIS: May I ask Dr. Everett whether he has tried modifying the

[illegible] but we recorded a few cases in which ovulation occurred

THE INFLUENCE OF CERTAIN ADRENERGIC AND CHOLINERGIC DRUGS ON URINARY GONADOTROPHIN OUTPUT IN AMENORRHŒIC WOMEN

A. C. CROOKE

THE beautiful series of experiments described today by Everett and his co-workers and by Harris suggest that the flood of gonadotrophin from the pituitary gland which stimulates ovulation is mediated by adrenergic and/or cholinergic impulses. We have investigated the effects of adrenergic and cholinergic drugs on the gonadotrophin output in man.

Material and Methods

I. The Drugs

The adrenergic drug used in these experiments was amphetamine sulphate (dexedrine). We chose this substance because we have sometimes observed that amenorrhœic women treated with amphetamine sulphate for obesity begin to menstruate, even when they fail to lose weight. The first instance of this was an out-patient Sister, aged 37, who had had amenorrhœa for 15 years. She began to menstruate after 6 weeks treatment with amphetamine sulphate but she failed to lose weight. She continued to menstruate regularly for three months while receiving treatment and stopped when treatment was suspended. In recent months we have used L-amphetamine (lævedrine) because Chance (1951) has stated that lævedrine causes ovarian hypertrophy in the intact, but not in the hypophysectomized rat, whereas dexedrine has no such action. We have used this drug orally in doses of up to 60 mg. daily.

The cholinergic substances which we have used are prostigmine and acetyl-beta-methyl-choline-bromide (amechol). We tried prostigmine because of its well-known action in causing

menstruation when used as a test for pregnancy. We have given it subcutaneously in doses of up to 1 mg. 4-hourly for 5 days. We have given amechol orally in doses of up to 200 mg. hourly for 24 hours and subcutaneously in doses of up to 25 mg. ½-hourly for 4½ hours.

II. Clinical Material

We chose 14 young adult women with amenorrhœa for at least one year. The youngest was 18 and the eldest 31 years Four had primary amenorrhœa. The remaining 10 had had amenorrhœa for an average of 4½ years, with a minimum of 1 year and 8 months, and a maximum of 8 years. All but 3 had previously been attending the fertility clinic, often for more than a year, and had had the usual routine investigations done.

III. The Urinary Gonadotrophin Assay

Twenty-four-hour samples of urine were treated by the kaolin adsorption method described by Dekanski (1949) and by Loraine (1950), and assayed by the mouse uterus weight method described by Klinefelter, Albright and Griswold (1943). The results were expressed in units of a standard prepared in our laboratory from urine of menopausal women These units correspond fairly well with the mouse units of other workers, and normal menstruating women excrete up to *15 units a day.*

Results

The results of treatment may be conveniently divided into *those observed by gonadotrophin assay and those produced* clinically.

We have carried out 24 experiments on 14 women but we have used lævedrine alone in only one experiment because we have rarely had any clinical response in women with such prolonged amenorrhœa. In this experiment there was no significant increase in the output of gonadotrophin. We have also carried out only one experiment with prostigmine alone and this again showed no significant change.

In the remaining 22 experiments we have used combinations of lævedrine with prostigmine or with amechol, or else amechol alone. We have failed to demonstrate an increase in the output of urinary gonadotrophin in 5 women treated intensively for short periods. They were first given 40 mg. of lævedrine by mouth and then 25 mg. of amechol subcutaneously ½-hourly for 4½ hours, or 12·5 mg. ½-hourly for 9 hours. Treatment for longer periods sometimes appeared to cause a significant rise in the level. More often there was a tendency for the level to rise gradually for a day or two after treatment was stopped, as shown in Fig. 1 (*a* and *b*).

Four of the patients had persistently high levels of gonadotrophin output but were otherwise clinically indistinguishable from the rest. Treatment did not cause any immediate change but there was a tendency to fall to low levels a few days after treatment ended.

One of these patients was observed to have a high level in May (Fig. 2*a*) and on several previous occasions, but in June she was found to have a level which was normal for menstruating women. It rose during treatment and fell afterwards (Fig. 2*b*) and she menstruated 3 days after finishing treatment. This 27-year-old woman had had amenorrhœa for 6 years.

Clinical Results

It is difficult to assess the value of the clinical results in a series of only 14 patients treated with three different drugs in different dosages for a maximum period of only 7 months. However, six of the ten women with secondary amenorrhœa for an average of 4½ years have menstruated, and two of the four women with primary amenorrhœa have also menstruated. These last two are perhaps of greatest interest. One, aged 31 years, has had anovular menstruation on three successive occasions within two days of finishing treatment. Each period has increased somewhat in amount and in length over the previous one. The other woman, aged 26 years, menstruated 17 days after finishing treatment. She had apparently ovulated within a few days of finishing treatment, as judged by the

menstruation when used as a test for pregnancy. We have given it subcutaneously in doses of up to 1 mg. 4-hourly for 5 days. We have given amechol orally in doses of up to 200 mg. hourly for 24 hours and subcutaneously in doses of up to 25 mg. ½-hourly for 4½ hours.

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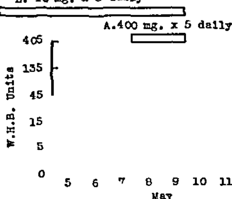
Results

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We have carried out 24 experiments on 14 women but we have used lævedrine alone in only one experiment because we have rarely had any clinical response in women with such prolonged amenorrhœa In this experiment there was no significant increase in the output of gonadotrophin. We have also carried out only one experiment with prostigmine alone and this again showed no significant change.

alone and amechol alone. Moreover, it seems that short periods of treatment lasting only a few hours are quite as

A.T.



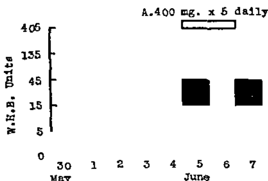


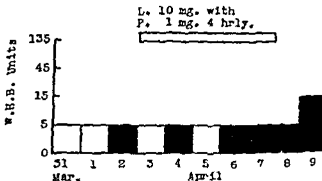
FIG 2.

A T Daily output of urinary gonadotrophins in a woman aged 27 with six years amenorrhœa treated with lævedrine (L) and amechol (A)

Daily output of urinary gonadotrophins in the same woman treated with amechol (A) only.

often followed by menstruation as are intensive courses lasting for five days.

examination of an endometrial biopsy specimen taken within an hour of commencing menstruation.



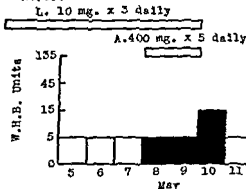


Fig. 1.

E.C. Daily output of urinary gonadotrophins in a woman aged 30 with four years amenorrhœa treated with lævedrine (L) and prostigmine (P).

M.F.C. Daily output of urinary gonadotrophins in a woman aged 20 with three years amenorrhœa treated with lævedrine (L) and amechol (A).

Menstruation has occurred in these eight women after prostigmine and lævedrine, amechol and lævedrine, prostigmine

terms of this standard, but they might equally be expressed as mouse units. We also take rather big steps from one concentration to the next in order to cover a wide range of concentrations. Three normal women whose output we have studied daily over one or more cycles, by this method, have shown very even curves. Generally it is consistently below 5 units till mid-cycle when it rises to 15 units. This corresponds well with what others have found.

Querido: I think we must have a number of experiments under controlled conditions, in the sense that nobody knows what is being injected. Sometimes women will be amenorrhœic for a couple of years, they come to the office, you only talk to them, and a month later they menstruate.

Crooke: That is true, and I think there is much more work to do [illegible]

therefore, a good control and it was some little time before I began to think that benzedrine might have something to do with menstruation.

Discussion

The significance of these preliminary observations must await confirmation from a larger series of patients but it seems unlikely that the high incidence of menstruation in this group is fortuitous and very unlikely that ovulation would have occurred unless the pituitary gland had been activated. It is difficult to see, however, why any drug acting on the pituitary gland should be effective in causing menstruation in women who are already excreting normal or even excessive amounts of gonadotrophin. It is possible that the cerebral stimulus to the ovarian cycle has failed and that there is never a sufficiently great surge of gonadotrophin to cause ovulation. The pituitary might then continue to secrete gonadotrophin at a constant slow rate, but as the ovary gradually fails the amount of gonadotrophin secretion would increase. Yet it seems from our results that even when it has reached menopausal levels it may still be possible to resume normal ovarian function as a result of adrenergic or cholinergic stimulation.

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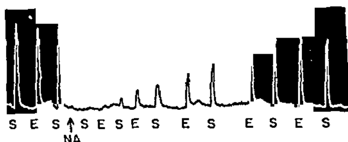
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DISCUSSION

Fig. 2 demonstrates the effect of an extract from a dog's hypothalamus before and after incubation with histaminase.



(4) The active agent is resistant to boiling with 6 N-hydrochloric acid in a water bath for one hour.

(5) The agent is not inactivated by crystalline trypsin prepared by our colleague Dr. P. Edman.



FIG. 2 Guinea-pig's ileum S 0·02 μg histamine (base) H and H_h 0·15 ml of an extract of a dog's hypothalamus before and after treatment with histaminase, respectively.

The smallness of the median eminence presents a hazard in these experiments. In cats the region excised and here referred to as the median eminence has a weight of 0·7–2 mg.,

THE OCCURRENCE OF HISTAMINE IN CEREBRAL REGIONS RELATED TO THE HYPOPHYSIS

G. W. HARRIS, DORA JACOBSOHN and G. KAHLSON

It is well established that the neurohypophysis contains histamine. This amine is a potent stimulant to certain glandular structures, for example the parietal cells of the gastric mucosa and the chromaffin cells of the suprarenal medulla It has been suggested that definite nerve fibres, referred to as "histaminergic," control the activity of effector cells by the liberation of histamine (Ungar, 1935; Ungar and Parrot, 1939; Kwiatkowski, 1943).

The aim of the experiments reported here was to investigate the occurrence of histamine in the hypophysis and in those parts of the brain which are linked up with the hypophysis by the hypophyseal portal vessels or by nerve fibres.

The active agent, here referred to as histamine, has the following properties:—

(1) It contracts the atropinized guinea-pig's small intestine.

(2) It is inactivated by the enzyme histaminase prepared from the pig's kidney.

(3) Its activity on the isolated intestine is antagonized by the antihistamine substance neoantergan. This test was performed as described by Roberts and Adam (1950) and is illustrated in Fig. 1. At the marks S and E, respectively, 0·01 μg. histamine (base) and 0·08 ml. of an extract of a dog's median eminence was added to a bath in which a piece of gut was suspended. After adding 0·1 μg. neoantergan to the bath, histamine and the extracted median eminence in the previously applied doses have no visible effect on the gut. Recovery of the sensitivity of the gut to equiactive doses of the standard histamine solution and the extract occurs at a similar rate.

of this extract is equiactive with 0·022 μg. histamine (base). On incubation with histamase the quick type of activity disappears. The histamine free extract now causes a slow type of contraction if the extract is left in the bath sufficiently long. The oxytocic activity in the rat's uterus is destroyed on boiling with 6 N-hydrochloric acid in the water bath for one hour (Fig. 4). It can thus be excluded that the oxytocic activity of the median eminence may simulate histamine-like activity in the assay on the gut.

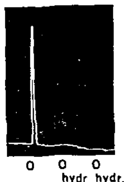


FIG. 4 Rat's uterus. O and $O_{hydr.}$ respectively, oxytocic activity of extract of the international standard powder before and after boiling with 6 N-hydrochloric acid.

Table I represents the histamine content of the total brain and some regions which are not specifically linked up with the hypophysis. In these regions and the total brain, extracted with trichloracetic acid, the histamine concentration is too low to be estimated with certainty. The figures for total brain confirm those published by Kwiatkowski (1943). Conspicuously high figures for histamine in nerves and peripheral ganglia have been recorded by Koshtojans *et al.* (1945) and by von Euler (1949).

Table II shows the histamine content of the anterior and posterior lobes of the pituitary gland, of the hypothalamus, of the median eminence and of sympathetic ganglia (in the cat superior cervical, in the dog cœliac). The tissues were

in dogs 5–12 mg., in pigs 10–15 mg. The median eminence of a dog may contain 0·1 µg. histamine. A proper assay and identification on the cats' blood pressure requires the equivalent of approximately 1·0 µg. histamine (base), five to ten times the quantity extractable from the median eminence of a dog. A sensitive piece of guinea-pig's gut readily responds to 0·01 µg. histamine. With a few exceptions the assay and identification was done exclusively on the gut.

The extraction of very small quantities of histamine also entails hazards. In experiments where 0·05–0·1 µg. histamine (base) was added to 10–20 mg. of minced brain, recovery was better with acid alcohol than with trichloracetic acid. With these small quantities recovery was inconsistent, varying between 35 and 100 per cent. It thus must be considered a possibility that the figures given here for the content of extractable histamine are lower than the amount actually present in the median eminence.

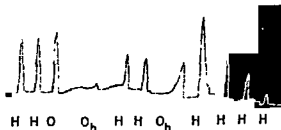


FIG. 3. Guinea-pig's ileum H 0·022 µg. histamine (base). [illegible] tional [illegible] after [illegible] r two [illegible] nutes

We have observed that extracts of the median eminence exert oxytocic activity on the rat's uterus used as described by Pamela Holton (1948). Obviously it must be ascertained whether the histamine-like activity of the median eminence on the guinea-pig's gut is in part due to this oxytocic agent. From the international standard powder of posterior pituitary lobe a solution 1 part in 1000 was prepared. In Fig. 3, 0·2 ml.

Table II

HISTAMINE IN μG./G.

TCA = trichloracetic acid   B = animal killed by blow on the head and exsanguinated   E = killed by electric shocks and exsanguinated

	Anterior lobe pituitary	*Posterior lobe pituitary*	*Hypothalamus*	*Eminentia mediana*	*Sympathetic ganglia*	*Extraction*	*Remarks*
Cats (9)*		5.7		16.0		TCA	B
Cats (5)	13	17.0	3.4	25.5	4.0, 4.2, 4.8, 5.0, 5.7	Alcohol	B
Cats (3)	0.26	1.7	4.3	4.2	6.0	Alcohol	B
Cats (3)	2.4	5.8	8.3	lost	5.2	Alcohol	B
Cats (6)	1.9	3.5	0.7	3.0	4.0	Alcohol	B
Cat	5.6	6.0				Alcohol	B, pregnant
Cat	27.0	54.0		13.0		Alcohol	B, pregnant
Cat	43.0	76.0			15.8	Alcohol	B, pregnant
Cat	4.5					Alcohol	B, 4 days starvation
Cat	3.4	<1.0	2.1			Alcohol	B, 5 days starvation
Cat	26.7	4.0	3.3		7.7	Alcohol	B, 7 days starvation
Dog				9.0		TCA	B
Dog	1.0	26.5	6.2	5.7		TCA	B
Dog	1.2		1.0	15.9	4.5	TCA	B
Dog	19.0	9.5		17.0	6.3	TCA	B
Dog	1.8	<0.4	<0.2	8.0	1.2	TCA	B
Dogs (2)	6.3	15.0	<0.4	30.0		TCA	B
Dogs (2)	5.4	13.6	4.0	12.5		Alcohol	B
Dogs (2)	7.0	12.0	2.0	20.0	9.5	Alcohol	B
Dogs (2)	8.2			12.0		Alcohol	E
Pigs (2)	0.1	1.2	0.2	9.0		TCA	E
Pigs (3)	0.24	7.0	0.14	11.0		TCA	E
Pigs (3)	1.0	3.0	0.75	3.0		TCA	E
Pigs (3)	0.12	2.8	0.12	2.3		TCA	E

*The figures in parenthesis indicate the number of brains pooled in preparing extracts

Table I

HISTAMINE IN µG./G IN DIFFERENT PARTS OF THE BRAIN (EXTRACTED WITH TRICHLORACETIC ACID)

	Total brain	*Thalamus*	*Nucleus caudat*	*Cerebellum*
Guinea pig	<0 1			
Guinea pig	<0 1			
Guinea pig	<0 1			
Cat	<0 04			
Cat	<0 03			
Cat	<0 03			
Cat	<0 06			
Cat	<0 06			
Cats (9)*			<0 2	
Cats (5)		<0 4	<0·6	<0 05
Dog		<0 3		
Dog		<0 05	<0 05	
Dog		<0 3	<0 1	<0 05
Dog		<0 2	<0 2	<0 2
Dog		<0 1	<0 1	<0 1
Dog		<0 1	<0 1	<0 1
Dogs (2)			<0 05	<0 03
Pigs (2)		<0 1	<0 06	
Pigs (3)			<0 04	<0 02
Pigs (3)		<0 05	<0 03	<0 02
Pigs (3)		<0 04	<0 04	
Calfs (2)			<0 02	
Calfs (2)		<0 1	<0 02	

*The figures in parenthesis indicate the number of brains pooled in preparing extracts

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DISCUSSION

LONG: Dr. Kahlson's paper suggests the possibility that histamine may be a specific stimulant for ACTH secretion.

KAHLSON: I don't know of any experiment which proves that histamine is a specific stimulant for the secretion of ACTH. There are guesses and indications. We would get nearer something resembling evidence [illegible]

or otherwise induce secretion in the anterior lobe.

LONG: The identity of the neurohumoral transmitter between the nerve endings in the median eminence and the cells is a matter of considerable interest. It has been suggested at one time, I think by Dr. Markee's group, that it was adrenergic in nature. It would be interesting if this were the case since it might allow for a reconciliation between the views expressed by Dr. Harris and ourselves.

[illegible]

immersed in acid alcohol or trichloracetic acid as soon as possible, 10–30 minutes after death. Confirming previous workers, the posterior lobe, with two exceptions, contains histamine in high concentrations. The anterior lobe, with a few exceptions, is also very rich in histamine. The hypothalamus, as a general trend, compares approximately with the concentration in sympathetic ganglia. The median eminence is conspicuously rich in histamine. The actual concentration in the median eminence proper may possibly be considerably higher than indicated in the table. In the first place, the histamine may be restricted to a minor region of the structure which was excised and extracted. Secondly, unknown proportions of the minute quantities of histamine originally present might have been lost during the processes involved in preparing the samples for the final assay on the gut. In several instances the histamine concentration of the median eminence is of the same order as in the gastric mucosa where histamine is believed to act as a stimulant to the parietal cells (Emmelin and Kahlson, 1944).

In contrast to sympathetic ganglia, the histamine concentration of the anterior and posterior lobes of the pituitary, the hypothalamus and the median eminence varies greatly in different individuals of the same species. The range of individual variation in the histamine concentration is narrower in the median eminence than in the anterior lobe. This fact might be taken to indicate the possibility that in the median eminence histamine may be related to nerve terminals from where histamine, dependent on the state of activity, is carried to the anterior lobe in quantities anywhere between minimal and maximal. It is possible that in the hypothalamus histamine is contained mainly in those neurones which terminate in the median eminence.

Much work remains to test these speculations and to see whether histamine actually has a specific effect on the anterior pituitary gland similar to its capacity to induce secretion in the parietal cells of the stomach and to liberate adrenaline from the suprarenal medulla.

THE INFLUENCE OF VARIOUS STEROIDS AND STEROID HORMONES ON THE HORMONAL ACTIVITY OF THE ANTERIOR LOBE OF THE PITUITARY GLAND—EXPERIMENTS IN HUMAN BEINGS

CHRISTIAN HAMBURGER and MOGENS SPRECHLER

THE aim of the investigations to be reported here has been to throw light on the interaction between the hypophysis, the gonads and the adrenal cortex in man by determinations of the urinary excretion of 17-ketosteroids (17-KS) and reducing corticoids during the administration of various steroid substances.

The experimental subjects were normal healthy persons and patients suffering from rheumatoid arthritis and other "collagen diseases," cancer of the uterus, and Addison's disease.

Twenty-four-hour-urine specimens were collected carefully and examined for 17-KS and for reducing corticoids The 17-KS were determined by the technique described by Hamburger and Rasch (1948), and the corticoids by Sprechler's method (1950), which involves the determination of the reducing property of the ketonic fraction of chloroform extracts of the urine specimens. (Usually the steroid excretion was determined for 4–5 days prior to the first injection, during the administration of the steroids, and for some days afterwards.)

The interpretation of the experimental results meets with several difficulties, the most important of which are the following.—

We presuppose that in women the urinary content of 17-KS reflects a function of the adrenal cortex, and only of this. This presupposition might be incorrect, as the human ovaries

[illegible] lethal dosages but [illegible]

substances the interpretation is difficult because these drugs have so many effects. One action which might be quite important in this connection was demonstrated by *Emmelin and Muren* in our laboratory. They found that some antihistaminic substances are potent liberators of adrenaline from the adrenal medulla.

As to histamine, I have seen some interesting papers where histamine [illegible] commented on as rather large, [illegible] it I [illegible] ies, [illegible] ices [illegible] line from the chromaffin cells It seems more accurate when using big [illegible] of ACTH to refer to histamine [illegible] such [illegible] were

VOGT: In answer to this last point, histamine releases ACTH even in the absence of the adrenal medulla, so I think that that is not the only possible mechanism.

There was one animal in that table which had a particularly high histamine concentration in the tuber, and another with a very high concentration in the anterior lobe. Do you know anything about these animals which made them different from the others? Their preceding history or something?

KAHLSON: No In future experiments we will try to correlate the mode of killing and the state just preliminary to death with the distribution of histamine in the regions under discussion.

In one case the experimental subject was a young man with homosexual tendencies. Œstrogenic substances were given partly therapeutically, that is in order to suppress the sexual libido, and partly experimentally. In the course of the 10 months' observation period, 185 24-hour urines were assayed for 17-KS, 120 of which were also assayed for corticoids. The pre-treatment level of 17-KS was normal for his age (15 mg./24 hr.). Single intramuscular injections of œstradiol benzoate in oily solution resulted regularly in a marked decrease to about 7 mg. 17-KS/24 hr. The pre-treatment level was reached in the course of about 12 days. Ten injections of 5 mg. œstradiol benzoate each depressed the 17-KS for about 26 days. At one period a combined administration of pregnenolone and œstradiol benzoate was given (see below).

Before the pre-treatment level was reached, ethinylœstradiol was given orally daily for 5 months, the daily dose being from 0·05 to 0·20 mg. As small an amount as 0·10 mg. a day suppressed the 17-KS excretion to, on the average, 6–7 mg./24 hrs. After discontinuation of this prolonged œstrogen administration, the 17-KS remained low for 8 days and increased gradually to reach the pre-treatment level 26 days after the last injection.

In contrast to the 17-KS, the corticoids did not change significantly during the œstrogen administrations. In some instances there seemed to be an increase immediately after the œstrogen, but during the long-term oral œstrogen ingestion the corticoids were somewhat below the average. The difference between the excretion pattern for corticoids (irregular fluctuations) and 17-KS (rapid return to pre-treatment levels) during the ultimate "period of recovery" was very impressive.

During the periods of decreased 17-KS excretion the testes were small and soft, and the erectile power and libido were also markedly impaired. Increased pigmentation of the nipples and of the skin in the genital region occurred, but otherwise there were no untoward reactions.

Single intramuscular injections of œstradiol benzoate (8–10 mg.) were given to 6 women. In three of the subjects

probably also produce androgenic substances and, therefore may contribute to the excretion of 17-KS. It has been shown however, that the amount of the total neutral 17-KS does no change after ovariectomy, and determinations of the tota neutral 17-KS must consequently be independent of the ovarian activity.

We assume that the excretion of reducing corticoids in both sexes reflects a function of the adrenal cortex only, and we think that no serious objections can be raised against this supposition.

Furthermore, we assume that among the hypophyseal hormones only ACTH stimulates the adrenocortical function, admitting, however, that it has not been definitely proved that the luteinizing hormone (LH) could not have a similar effect.

In normal (i.e. non-castrated) men the 17-KS represent metabolites both from testicular and adrenocortical hormones, and changes in the 17-KS excretion can, therefore, be due either to an altered hormonal activity of the testes or of the adrenal cortex. If, however, both 17-KS and corticoid determinations are carried out, we assume that a decrease or increase of the 17-KS, with constant corticoid excretion, indicate a decreased, or an increased testicular activity respectively.

The evaluation of the steroid analyses may furthermore be complicated by the fact that the administration of steroid substances might alter the metabolic breakdown of the endogenously produced steroids.

Summarizing, we presuppose that changes in the corticoid- [illegible] s [illegible] e changes in the 17-KS without corresponding [illegible] corticoids in normal men, are related to the gonadotrophic hormones.

Admitting these difficulties for the interpretation of our analytical results, we shall present some cases which show the effects of steroid hormone administration on the 17-KS and corticoid excretion.

inhibitory effect of œstradiol. In order to see whether pregnenolone in man would protect the hypophysis against œstradiol, the first-mentioned patient was treated with large doses of pregnenolone (intramuscular injections of 500 mg. microcrystals and three ·300 mg. linguets) and one intramuscular injection of œstradiol benzoate. In spite of the pregnenolone administration, the 17-KS decreased as after œstradiol benzoate alone. In this case pregnenolone did not interfere with the inhibitory effect of œstrogen on the gonadotrophin production.

It is well known that testosterone and its esters are converted to, and excreted as, 17-KS. After the high excretion of 17-KS a period of decreased excretion usually occurs. As this "negative phase" was found also in women treated with testosterone propionate (Hamburger and Kaae, 1949), it was thought that the ACTH production was inhibited by testosterone. We can now add 3 new cases (male subjects) in which both corticoid and 17-KS determinations have been carried out. The 17-KS and the corticoids in a 34 years old man, treated at first with testosterone valerate crystals, and afterwards with testosterone propionate in oily solution, showed that, after the peaks in the 17-KS, marked suppression took place, and there seemed to be some depression of the corticoid excretion also. In a second case no suppression of the corticoid excretion occurred, and the decrease in the 17-KS was slight, although unquestionable. Very high doses of testosterone propionate in oily solution were injected intramuscularly to a 58 years old man. One hundred mg. were injected daily, and after a few days of treatment there was a marked inhibition of the corticoid excretion.

In 8 cases the 17-KS and corticoid excretion were determined before, during and after the administration of *cortisone acetate*. In two male patients the corticoids increased in both cases, indicating an excretion of metabolites from the exogenous cortisone. In the younger patient the 17-KS decreased, in the other they increased during the cortisone administration, and in a third case (a woman 56 years old) 2,000 mg.

both corticoids and 17-KS were determined, in the others only the 17-KS. In half of the cases the 17-KS excretion increased somewhat; in the others it was more or less diminished. The changes cannot be regarded as significant. The slight decrease in the corticoid excretion did not exceed the normal daily variations.

A surgical male castrate, 50 years old, received a single intramuscular injection of œstradiol dipropionate. Both corticoids and 17-KS decreased slightly, but probably not significantly.

The effect of injections of *progesterone* was shown in a healthy male 33 years of age. The total dose of 300 mg. progesterone given intramuscularly in oily solution, caused a marked decrease in the 17-KS excretion and a slight decrease in the corticoids.

Pregnenolone was given to several experimental subjects. In the first case, a 33 years old normal man, two intramuscular injections of 100 mg. pregnenolone (suspension of micro-crystals) caused a moderate, but unquestionable decrease of the 17-KS. In another healthy man, daily oral administration of pregnenolone linguets (50–100 mg.) did not change the 17-KS excretion, whereas a moderate decrease took place after intramuscular injection of a total of 250 mg. In a young woman of 25, two injections of 100 mg. each caused a gradual decrease in the 17-KS excretion. The corticoid excretion showed marked fluctuations, but the average daily excretion was the same before and after the injections. In a woman suffering from chronic rheumatoid arthritis, a total amount of 1,400 mg. pregnenolone acetate crystals suppressed the 17-KS markedly, but in another arthritic patient 1,600 mg. of the same preparation gave rise to an increased steroid excretion, especially of the corticoids. As the injections of the large crystals were very painful, and the patient developed a fever, we believe that the increased steroid excretion must be regarded as a "stress reaction."

It has been claimed (Selye, 1942) that pregnenolone in animal experiments could protect the hypophysis against the

inhibitory effect of œstradiol. In order to see whether pregnenolone in man would protect the hypophysis against œstradiol, the first-mentioned patient was treated with large doses of pregnenolone (intramuscular injections of 500 mg. microcrystals and three ·300 mg. linguets) and one intramuscular injection of œstradiol benzoate. In spite of the pregnenolone administration, the 17-KS decreased as after œstradiol benzoate alone. In this case pregnenolone did not interfere with the inhibitory effect of œstrogen on the gonadotrophin production.

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It has been claimed (Selye, 1942) that pregnenolone in animal experiments could protect the hypophysis against the

of the corticoid excretion occurred in some cases. The fact that the 17-KS, but not the corticoids, were depressed in one of the normal males, might indicate that the gonadotrophin production was also inhibited.

Cortisone acetate and *deoxycorticosterone acetate* seemed to *inhibit the ACTH production.* From our experiments it could not be decided whether any changes in the gonadotrophin production occurred.

The inhibitory effects of the steroid substances on the hypophyseal production of "-trophic" hormones are fully reversible.

General Conclusions

In human subjects the ACTH production was inhibited by all the steroids examined—with the exception of œstrogens.

The gonadotrophin production is inhibited by œstrogens, and probably also by progesterone and testosterone. The effects of the other steroids on the gonadotrophin production were not clarified by the present experiments.

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DISCUSSION

[illegible]

the testis as it is in laboratory animals, but we learned that all levels of

cortisone acetate given in the course of 19 days, caused no significant changes in the steroid excretion.

Quite recently we have followed the 17-KS excretion during cortisone administration in a girl 13 years of age with adrenocortical hyperplasia. She excreted about 90 mg. 17-KS per day. During the cortisone acetate injections (50–100 mg. per day), the 17-KS decreased, and 2 days after the last injection the excretion was depressed to 4·5 mg./24 hr., which is normal for her age. Then the 17-KS increased rapidly and in the course of 7 days reached 54 mg.

The last case to be reported here is a female Addisonian patient during the administration of *deoxycorticosterone acetate* injections (10 mg. in oily solution daily). The total reducing corticoids increased during the injections, probably as the result of metabolites from the injected DCA, whereas the water-soluble fraction from the benzene-water partition decreased somewhat, indicating a diminished endogenous corticosteroid production. The 17-KS did not change significantly.

From these investigations we believe it justified to draw the following conclusions:—

As œstrogenic substances suppress the 17-ketosteroid excretion markedly only in the presence of the testes, and as no significant changes in the corticoid excretion were noticed, *œstrogens* seem to *inhibit the gonadotrophin production* but to have *no effect on the ACTH production.*

Progesterone caused a marked 17-KS depression and a slight corticoid depression in a normal man, presumably due to a *marked gonadotrophin inhibition* and *a slighter ACTH inhibition.*

As *pregnenolone* caused a depression of the 17-ketosteroids in some of the females as well as the male subjects, it seems to *inhibit at least the ACTH production.* It could not be decided whether the gonadotrophin production was influenced. Pregnenolone did not "protect" the hypophysis against the gonadotrophin inhibition produced by œstrogens.

Testosterone inhibits the ACTH production, as a suppression

THE CO-ORDINATION OF HYPOTHALAMIC VEGETATIVE CONTROLS

G. C KENNEDY

THERE is a great deal of experimental evidence that stimulation or destruction of well defined areas of the hypothalamus causes disturbances of vegetative functions, and that this region plays a vital part in the maintenance of homoeostasis. But with the exception of the supra-optic nuclei, none of the so-called nuclei of the hypothalamus has been implicated with certainty in any specific control. And the absence of reliable methods of studying degenerating nonmyelinated fibres means that we have almost no knowledge of the anatomical consequences of the lesions which have such striking effects. So we must deduce what we can of the relations of hypothalamic areas one with another, and with other parts of the central nervous system, from disturbances of physiological behaviour. I should like, therefore, to describe an investigation of the relation between the effects of a variety of hypothalamic lesions which appear to disturb the regulation of energy exchange in the rat.

Brobeck, Tepperman and Long (1943) showed that the hypothalamic mechanism controlling food intake was most vulnerable in the region of the ventro-medial nuclei of the tuber cinereum and that quite small bilateral lesions in this area would regularly produce enormous obesity. The obesity is characterized by a striking increase in food intake during the stage of development, although later the food eaten returns to normal levels. This hyperphagia, as Brobeck has called it, is clearly the chief cause of the obesity, as little evidence has been found in the obese rats of any alteration in intermediate metabolism or activity which would be sufficient to explain the rapid deposition of fat. The limitation of hyperphagia

are inferior, that is those which show certain fibrotic changes, are more likely to be damaged by a steroid substance than are more normal testes.

During the last year, with Dr. William Maddock of Detroit, we have had occasion to treat eight men with chorionic gonadotrophin, and have followed them with testicular biopsies before and after treatment, and [illegible]

were also treated with chorionic gonadotrophin. There was no change whatsoever in their 17-ketosteroids, which I think bears on the point you have made about LH, at least chorionic gonadotrophin LH, not having an effect on the adrenal cortex.

NOWAKOWSKI: I would like to ask Dr. Hamburger about the cortical steroid excretion in the case of adrenogenital syndrome.

HAMBURGER: The cortical steroid excretion is being determined in this case, but it is a very recent case, and the results have not appeared yet.

food more attractive, such as serving it as a wet mash rather than dry, or mixing it with olive oil or nut oil, usually make the fat rat fatter still, but never affect the weight or food intake of the normal rat. In fact, however one disguises the diet, palatability does not seem to affect the calorie intake of the normal rat, but it appears to be a major determinant of the amount the obese rat eats, and consequently of how fat it becomes. This emphasizes two different aspects of satiation —a calorimetric one which is dominant in the intact animal and damaged by the lesion, and a conditioned discriminative one which becomes more important with the accumulation of fat after operation. Because of the wide variety of conditioned reflexes which affect this second aspect of satiety it is suggested that it is a cortical function. Finally, there is a third familiar aspect of feeding behaviour which has not been considered—hunger, the urge to eat induced by the deprivation of food. It is conspicuous in all our experiments that no diet, however unattractive, ever reduces the weight of an obese rat below that of its control. As their weights approximate, their feeding behaviour becomes identical. Moreover, we have not encountered any hypothalamic lesion which will

be summed up in a simple diagram (Kennedy, 1951) —



This represents food intake as being governed by a balance between hunger and satiety, satiety having the two major aspects I have described. The release of hunger from hypothalamic inhibition causes hyperphagia. As fat accumulates, a threshold is eventually reached when the damaged hypothalamic mechanism is again able to inhibit hunger. As this

with the accumulation of fat deposits is not due to recovery from the effect of the hypothalamic damage, as hyperphagia can be re-induced subsequently following a short period of starvation which removes some of the fat. This conditioning of feeding behaviour by the amount of body fat is very striking and will be referred to again.

Hyperphagia is not merely undiscriminating voracity. A hyperphagic rat may eat three times as much of its normal diet as its unoperated litter mate, but when offered an unappetizing food such as a mixture of its usual diet with powdered kaolin, it refuses it completely. Yet the unoperated rat immediately increases the bulk of food it eats to compensate more or less exactly for the presence of the diluent, so that it obtains the same calorie intake as before (Kennedy, 1950). So strong is this urge to eat for calories that the normal rat can be made to quadruple the bulk it usually eats in order to maintain its nutrition. The discrimination shown by the obese rat is conditioned by the amount of fat in its depots. Soon after operation it may eat more of a kaolin mixture than a control animal and continue to get fat on it, but when it is obese it appears to prefer to live on its fat and it may eat nothing for as long as ten to fourteen days. Naturally, it loses weight, and as it does so its food intake gradually increases, until finally it eats precisely the same amount as the control animal and its weight becomes steady. The weight level at which this occurs is characteristic for the animal and the particular diet. By varying the proportion of kaolin in the mixture one can make a fat rat take up a different weight for each proportion. The effect is not restricted to bulky, adulterated diets. Almost any method of making food less attractive, such as feeding it dry, with a restricted fluid intake, or adding unpleasant flavours, affects the food intake of the obese rat more than that of the normal one. Miller, Bailey and Stevenson (1950) have reported similar effects from adding quinine to the diet and from using mechanical devices to restrain the animal from reaching its food easily. The converse is also true, that things which apparently make

thalamic hyperphagia from those areas responsible for other hypothalamic symptoms, and secondly, to demonstrate the integrity of the co-ordination of vegetative functions after damage to these hypothalamic areas.

First, the relation between food and water intake (Bruce and Kennedy, 1951*a*). In general, on the stock diet we use, a normal rat drinks about the same weight of water as it eats of food, and day to day variations in the one are mirrored in similar changes in the other. Strominger showed that this relation is usually preserved in hyperphagia and we have confirmed this. Now by careful placing of hypothalamic lesions one can produce hyperphagia alone, or combine it with diabetes insipidus, or produce diabetes insipidus alone. Richter (1938) has given reasons for regarding the limiting water intake a day in diabetes insipidus in the rat as about equivalent to the body weight of water. We have produced a number of hyperphagic rats in which the water intake on a normal intake of food was of this order but when eating is unrestricted, such an animal doubles its food intake, and its water intake increases proportionately so that it may exceed twice the animal's body weight a day. Such animals have severe damage to the hypothalamic centres concerned in the control of both food and water intake, yet the "co-ordination" of food intake and water intake is still preserved. It can hardly be argued that the hypothalamus is essential for the co-ordination. The more likely explanation is suggested by the fact that the apparent co-ordination can easily be upset in either the normal or the operated animal by changing the chemical composition of the diet, so as to vary the excretory load on the kidney. The usual relation between food and water intake is probably due merely to the fact that any change in the amount eaten naturally changes the excretory load on the kidney and therefore the volume of urine, which is ultimately the chief determinant of the water intake. As the sensitivity to osmotic diuresis is preserved in diabetes insipidus, the effect is still seen.

Clinically, the association between obesity and changes in the sex cycle is familiar and early reports on experimental

stage is approached, hunger becomes weaker and the cortical aspect of satiation becomes more important.

This leads us to the inter-relations of the hypothalamic mechanisms concerned in the regulation of energy exchange. Dr. Brobeck has suggested that the hypothalamus integrates all the processes of energy exchange. As he has pointed out (1946) an animal must constantly submit to changes in diet, in work and in environmental temperature, and the precision with which it always manages to balance its energy equation is suggestive of central co-ordination. He has made the intriguing suggestion that the basis of the integration is the effect which all these environmental changes have on temperature regulation. Dr. Strominger (1947), also of Yale, has suggested that there may be an inter-action between the tuberal centres concerned in these effects and the supraoptic centres concerned in the control of water balance. I should first like to make the general point that even if one grants the existence of central co-ordination, it need not be hypothalamic. The central control of food intake must involve more than one level in the central nervous system—it probably involves a much more complex hierarchy than the simple one I have suggested. And if one considers the analogy of the motor system, the more complex co-ordinated patterns of movement are represented in the cortex, while the simpler more specific effects on individual muscles are elicited by stimulation or destruction of lower levels, such as the motor tracts or anterior horn cells. The crowding together of the descending pathways makes them more vulnerable to small lesions than the cortex, and a similar effect might explain the relative ease with which one can elicit symptoms by damage to the hypothalamus as compared with, say, the frontal cortex. The anatomical simplicity and comparative primitiveness of the hypothalamus make it seem more likely that it should be concerned chiefly with reflex rather than *co-ordinating* functions.

Our experimental approach has been, first, to distinguish the anatomical area concerned in the production of hypo-

itself perfectly well on half that at 94°F. And this is a steady state, maintained indefinitely and not altered by subsequent acclimatization. So far as we can judge, the rats remain just as active as at the lower temperatures. Now the hyperphagic rat adapts to these conditions equally well and shows no evidence that its temperature regulation is disturbed. It maintains its previous rate of growth, but does it by eating 10 g. a day less, as the normal rat does. I think therefore that one can draw two conclusions—that the satiety mechanism is not directly affected by environmental temperature, and that it is concerned in regulating the excess of food intake over current needs, not the absolute level of intake. If food is removed from the body again after absorption, as it is for example in lactation, the hypothalamus appears to take no account of it. Normal rats treble their food intake during maximal lactation, but the growth of their litters reflects the re-export of two-thirds of the food they absorb. So the high absolute food intake is not inhibited by the hypothalamus. It is difficult to see, on thermodynamic grounds, how the body could be protected against an excess of heat and plethora of fat by the same centre. Food does not become the concern of the temperature regulating mechanism until it has been burned and has yielded its energy as heat. But it is clear from our feeding experiments that the chief determinant of the activity of the satiety mechanism is the level of fat in the depots. And as the potential energy of all this material is intact, sensitivity to its presence could only be achieved by a chemo-sensitive centre, presumably reacting to changes in blood metabolites secondary to the presence of the fat. Measurements of fat turnover have been equivocal, but just as the fat accumulates in the depots as a result of an excess of available food in the circulation, I imagine it might in turn raise the level of blood metabolites. There certainly is an increase of blood fat, for example, in the obese rat. I suggest that the tuberal nuclei of the hypothalamus must constitute a satiety centre with chemo-sensitive neurones analogous to V 's osmo-receptors or the CO_2-sensitive cells of the re

hypothalamic obesity stressed the occurrence of simil associations. It is, of course, difficult to isolate individu hypothalamic areas exactly, and just as it is possible to pr duce diabetes insipidus in association with obesity, so also variety of sex effects may be produced. A proportion operations carried out with the intention of producing obesil fail, although it can be shown that the lesions are usually ver near to the correct area. A comparison of the sex effect produced by these lesions has shown that they are identic with those in the obese animals (Bruce and Kennedy, 1951b

Finally, the relation between obesity and the control c body temperature. Brobeck (1948) has summarized his hypo thesis of food intake as a method of temperature regulation by saying that the animal eats to keep warm and stops eating to prevent hyperthermia. He considers that it controls it food intake by sensitivity to the heat released during metabolism. I shall try quickly to outline our reasons for disagreeing with this view and our evidence for a contrary hypothesis.

Brobeck has shown that rats exposed to temperatures of over 90°F. refuse food, lose a good deal of weight and become pyrexial. He suggests that the refusal of food is the primary effect and that the loss of weight is due to the animal calling on stored energy reserves. If that is so, the rat appears to derive no advantage from restricting its intake of exogenous calories, because it replaces this with endogenous food, which of course is just as pyrogenic. And it does not, in fact, succeed in preventing hyperthermia. In any case, it appears just as likely that the catabolism associated with fever is the primary effect and the animal refuses to eat because it is already deriving all the food it can handle from the breakdown of its own tissues. Because of this difficulty in interpretation, we have tried to avoid causing fever in our animals by stepping up the rate of ventilation in our hot room. It is quite easy to do this and still to reduce the heat lost by the animal to its environment very considerably. We have found that a rat which eats about 20 g. of food a day at 75°F. can maintain

itself perfectly well on half that at 94°F. And this is a steady state, maintained indefinitely and not altered by subsequent acclimatization. So far as we can judge, the rats remain just as active as at the lower temperatures. Now the hyperphagic rat adapts to these conditions equally well and shows no evidence that its temperature regulation is disturbed. It maintains its previous rate of growth, but does it by eating 10 g. a day less, as the normal rat does. I think therefore that one *can draw two conclusions—that the satiety mechanism is not* directly affected by environmental temperature, and that it is concerned in regulating the excess of food intake over current needs, not the absolute level of intake. If food is removed from the body again after absorption, as it is for example in

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centre. If this be granted, little central co-ordination of the different aspects of energy exchange need be postulated. Certainly we have been able to find no evidence of such co-ordination by the hypothalamus.

Although these experiments are concerned with a hypothalamic control which is probably not mediated through the pituitary, I think they are germane to the present discussion because they illustrate what may be one general type of hypothalamic reflex concerned in homoeostasis, involving direct chemical sensitivity to a disturbance of the internal environment.

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DISCUSSION

BROBECK: Our suggestion that food intake is controlled as a function of temperature regulation is only a hypothesis, and I think if it has any value it is only in stimulating experiments in this field. The hypo-

[illegible]

regulation. So I think some of the points raised by Dr. Kennedy [illegible]

be clarified by thinking in terms of SDA rather than the total caloric value of the food. For example, we know that during growth and during lactation the so-called SDA is less, and it would presumably require a larger quantity of food to liberate a given amount of heat in a lactating animal than it would in a normal animal.

Dr. Bal Anand in our laboratory has recently found that lesions in [illegible]

and he finds, as Dr. Kennedy has described, that when these lesions are [illegible]

first made our lesions to produce obesity, the animals never eat again. Following lesions ahead of or behind this area, the rats may show for a few days a fall in food intake, which eventually returns to normal. Dr. Anand has done the same sort of experiments in the other plane, [illegible]

on the other side there is no further food intake and the animal dies of starvation. The paper has been accepted by the *Proceedings of the Society for Experimental Biology and Medicine* (and published in completed form in the *Yale J. Biol Med*, 1951, 24, 123).

Kennedy It is surprising that one can localize this area because, as Dr. Brobeck has himself probably found, if one just misses the right place in trying to make an animal fat one gets acute anorexia, and, as he says, such animals always die. What puzzles me about it, and why I said specifically "chronic anorexia" was that I've never seen an animal go on as in Simmonds' disease or in anorexia nervosa, and live on a [illegible]

Brobeck: I think I can answer that, although not completely. The lesion in the region marked $\pm$ in our diagram led to a reduced food intake for fairly long periods of time, and there was often in such rats a complete absence of eating for a day or two after operation. I suppose that the popularity of lobotomy operations and the prevalence of obesity, taken together, would make one interested in the possible usefulness of this type of lesion; but at the moment the result is nearly an all or nothing response. As Dr. Kennedy said, nearly all of us that have worked

centre. If this be granted, little central co-ordination of the different aspects of energy exchange need be postulated. Certainly we have been able to find no evidence of such co-ordination by the hypothalamus.

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DISCUSSION

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THE INFLUENCE OF ENVIRONMENTAL CHANGES ON THE PITUITARY*

S. ZUCKERMAN

THE adaptive reactions of an animal in response to changes in its environment are mediated through the nervous and endocrine systems. The fact that the reactions are adaptive is, by definition, an indication of their co-ordinated character, and this in turn implies, first, that the activities of the nervous and endocrine systems are integrated, and second, that the endocrine system is responsive to environmental changes. On the other hand (and with the exceptions of the adrenal medulla, neurohypophysis and possibly the islets of Langerhans) such evidence as points to the existence of a secreto-motor innervation of the endocrine organs is both controversial and slight, and the way in which the environment influences the endocrine system is consequently little understood It is, however, widely assumed that the hypothalamus, the so-called head-ganglion of the autonomic nervous system, plays an important part in the process.

The environmental factors which influence the endocrine organs may be "physical" in the sense of an alteration in hours of daylight, or "social" in the sense of stimulation deriving from the group of which an animal is part. Some changes appear to be mediated through the sensory modality of touch—for example, the stimulation arising from coitus (or pseudo-coitus) which, in the rabbit or ferret, results in ovulation; stimulation of the reproductive tract which in the rat leads to pseudopregnancy; the pressure-pattern of the eggs which determines the number of eggs that are laid by a bird in a single clutch; and the nipple-stimulation on which

*This paper was not given at the Colloquium, as the author was absent through illness [*Ed*]

with hypothalamic lesions have found that some lesions have produced anorexia, and I had been under the impression that this was because the lesions were large. I attempted to persuade Dr. Anand of this when he first noted these animals, but he began working with smaller and smaller lesions and finally was able to localize the effective lesions very precisely. A large [illegible]

kaolin stuff that the others will. Has anybody experimented peripherally by destroying the taste buds and the sense of smell in these animals to see if this prevents overeating and obesity? I would think it is worth while.

KENNEDY: I don't know whether anyone has done this. We certainly haven't done it. And I quite agree that it ought to be done.

BROBECK: Another type of experiment that should be done (Dr. Kennedy may have done this, but we haven't) consists in putting these animals on a kaolin diet or something like that before operation, and then making the lesions to see if under such conditions the animals might not eat larger quantities of a diet to which they were accustomed. There is no doubt that changing the diet disturbs them, and whether they would eat these same diets if they were used to them as normal animals, we don't know. Do you know?

KENNEDY: No, I don't—although we have gone on giving this sort of diet for three months or so, and they don't get reconditioned to it during that period.

[illegible] by force feeding?

but when the lesions are small enough there is no obvious sleepiness.

PARKES: The animals won't drink if they don't eat, will they?

BROBECK: No. We haven't done the really careful measurements of water intake required to distinguish between evaporation and the lowered water intake of a fasted animal, but it is my impression that, like any fasted animal, they drink little or no water.

KENNEDY: I would like to ask Dr. Brobeck what happens to their temperature regulation, because the acute ones that I've seen have all been hypothermic.

BROBECK: With large lesions?

KENNEDY: Yes.

BROBECK: That's a common observation. Dr. Anand did measure rectal temperatures in a few of these, and found them to be normal. We keep our animals at a temperature of about 82°, so that there is not much cold stress.

A priori, it would therefore seem a relatively simple problem to track impulses from the retina to the point of effective stimulation of the adenohypophysis. In practice, attempts to do so have revealed little more than that neither the integrity of the visual cortex nor mid-brain is essential to the response (Clark, McKeown and Zuckerman, 1939). Moreover, uncertainty about the innervation of the adenohypophysis makes it unlikely that this kind of experiment can provide more than a partial answer to the problem. Further examples of enquiries that have led to inconclusive and conflicting results are those that have been designed to discover the pathways along which impulses, initiated apparently in the reproductive tract, travel in order to stimulate the pituitary, so causing either ovulation (e.g. the rabbit) or pseudopregnancy (e.g. the rat) (for general review see Harris, 1948*a*). In spite of our increasing understanding of the trophic control which the adenohypophysis exercises on other endocrine organs, it is only too clear that we know relatively little about the means whereby the activity of the endocrine system is related to a continually changing environment.

Obviously an essential key to this problem is an understanding of the ways in which the adenohypophysis, as opposed to other endocrine organs, is influenced by the stimulation of different bodily receptors. Another is a clearer understanding of the hormonal interaction of the adenohypophysis and the endocrine organs it stimulates, which reveals itself in the regulation of the *milieu intérieur* of the body. In general we conceive of the pituitary as controlling a number of distinct functions by means of a number of specific hormonal secretions. When, as it were, one tap is turned, the gland elaborates and/or secretes more gonadotrophin; when another, more ACTH or growth hormone. But the conditions which activate a given tap do not necessarily turn it the same way, or at the same relative time, in all species, or even in the two sexes of one species For example, an increase in the amount of illumination to which the ferret is exposed daily stimulates the production of gonadotrophin, and induces heat, whereas

the maintenance of milk-secretion at least partly depends. Others are mediated through the visual and auditory modalities, and comprehend such reactions as the increased secretion of adrenaline following a frightening noise; the induction of breeding activity in an anœstrous ferret as a result of exposure to illumination during the hours of darkness; and the egg-laying of an isolated pigeon which is shown its image in a mirror. The chemical changes in pond water which are said to initiate spawning in the common frog (Savage, 1935), and which presumably act on chemo-receptors in the skin, can be regarded as appertaining to yet another sensory modality.

With the possible exception of responses in which the adrenal medulla, the neurohypophysis, and the parathyroids may be concerned, the adenohypophysis, because of the trophic control which it exercises, is presumably a constant intermediate link in the environmental control of the endocrine organs. Thus exposure to artificial light during the anœstrum will not induce heat in a ferret deprived of its pituitary, any more than it will in one that is spayed. The chain of events in reactions of this kind is presumably (*a*) stimulation of one or more sets of receptors; (*b*) the transmission of afferent impulses to the thalamus, hypothalamus and cortex; (*c*) the initiation or modification of hypothalamic activity by thalamus and cerebral cortex; (*d*) excitation of adenohypophysis; and finally (*e*) differential activation of peripheral endocrine organs.

Attempts to describe and elucidate the links in this chain have focused mainly on two issues: (1) the control exercised by the adenohypophysis on other endocrine organs, and (2) the possible way in which the hypothalamus influences the adeno-, as distinct from the neurohypophysis, with which its interaction is fairly well understood. Other aspects of the process have been studied to a much lesser extent, and such investigations as have been carried out have provided only equivocal results. For example, the gonadal (œstrous) response of an anœstrous ferret to artificial illumination appears to depend in the first instance on retinal stimulation.

adrenal cortex and thyroid. Correspondingly the activation of the gonads (e.g. of ferrets, moles, etc.) at the start of the breeding season is associated with an increase in the size of the adrenal cortex and also with increased activity of the thyroid. Similar changes occur in the thirteen-lined ground squirrel (Moore, Simmons, Wells, Zalesky and Nelson, 1934; Zalesky, 1934) and are associated with a seasonal increase in the weight of the pituitary gland and an alteration in its cellular constitution. The thyroid is also said to enlarge during the breeding season of certain types of bird (see Selye, 1947).

The obvious inference from these facts is that whatever the stimulus (e.g. light in the ferret) that triggers the secretion of gonadotrophin at the start of the breeding season, it also sets in train other functions of the pituitary.

It may, however, be noted that the adrenal cortex hypertrophies in the thirteen-lined ground-squirrel, a species which does not react during hibernation to increased illumination, when its ovaries are activated during the anœstrum by gonadotrophin (Moore *et al.*, 1934). The animal does not possess an X zone, so that the enlargement of the adrenal cannot be regarded as a direct effect of the treatment on this cortical zone. Unless the gonadotrophin used in the experiment contained ACTH, this observation suggests, therefore, that whereas the activation of the gonads is a direct effect, that of the adrenals is in some way mediated indirectly (e.g. as a result of stimulation by gonadal hormones (see below).

2. The Parallelism of Ovarian and Adrenal Changes

The adrenal cortex enlarges in frogs (Stilling, 1898) and pigeons (Riddle, 1923) at the time of ovulation. Enlargement of the cortex also occurs at œstrus in the mouse (Masui and Tamura, 1926) and rat (Anderson and Kennedy, 1932; Bourne and Zuckerman, 1941*a*).

There are three possible explanations for this parallelism: (*a*) that the activated gonads directly stimulate the adrenals; (*b*) that at the times concerned the adenohypophysis secretes

it is the reverse condition of decreasing hours of daylight that heralds the breeding season in, say, deer and sheep. Again, while both the female and male ferret are sensitive to light, the female comes into œstrus at some time between the middle of March and April, whereas the male may begin to show signs of spermatogenesis early in December, well before the winter solstice. And in many mammalian species the sexual and reproductive activities do not appear to be influenced by light at all.

Many people have found it difficult to reconcile the conception of a number of separate hormonal taps working independently (and controlled reciprocally by the specific hormonal secretions they stimulate, by what is sometimes called a "push and pull mechanism"), with the fact of a much smaller number of distinctive secretory elements in the adenohypophysis. This difficulty is, however, hardly worth emphasizing in the face of our general ignorance about the mode of action of pituitary hormones at the cellular and molecular level. What is more important from the point of view of this discussion is the fact that the overlap in function of manifestly pure pituitary extracts (e.g. pure growth hormone has galactopoietic properties; while pure luteotrophin is lactogenic) also seems to qualify the belief that the number of distinct pituitary secretions is as many as the identifiable functions the gland controls. The primary purpose of this paper is therefore to draw attention to a few instances in which the activation of the pituitary by changes in the environment is simultaneously associated with the stimulation of more than one of the endocrine organs which it controls. Reference is also made to the likelihood that the same environmental stimulus may, under different physiological conditions, affect the pituitary in different ways.

1. The Activation of Endocrine Organs by Light

Many mammals with annual breeding seasons hibernate during the winter, and hibernation is as a rule associated with regression not only of the gonads, but also of the pituitary,

occurrence of some "inherent rhythm" in its function which persists after the removal of the pituitary.

Grollman's suggestion that the increase in the size of the adrenal cortex at the time of follicular rupture is merely a manifestation of enhanced bodily activity is too general to be assessed in the light of the available facts.

3. The Influence of Environmental Factors on Lactation

Rats kept in the dark do not grow as well as those which live under normal laboratory conditions. This observation applies both after puberty (Browman, 1940) and also to the first 18 days of life when both the doe and litter are kept in total darkness (Eayrs and Ireland, 1949). The delayed growth of the litter is associated with a decrease in food-intake by the doe. On the other hand, the depression of growth in normal non-lactating adult females kept in darkness is not associated with any change in the amount of food eaten.

In a series of experiments designed to explain these facts, Eayrs (1951*a*, *b*) has observed that growth is also retarded in the young of blinded females kept under normal conditions of daylight and darkness A comparison of the rate of growth of litters belonging to normal does that were allowed only a limited amount of illumination daily showed clearly that the growth of a litter is far more sensitive to changes in the conditions affecting the mother than it is to changes in the amount of illumination to which the young are subjected. Further tests showed that the pattern of a doe's activity is not significantly altered in the dark In view of this observation and the fact that the food-intake of mature non-lactating rats kept in darkness is not impaired, it follows that the depressed growth-rate of suckling young kept in the dark is due to a falling-off in the doe's powers of lactation, which may secondarily lead to a decline in food-consumption.

In view of the fact that they do not eat less, Eayrs suggests that the decline in growth of mature rats kept permanently in the dark may be associated with a fall in the level of

and releases ACTH as well as gonadotrophin; and (*c*) that as Grollman (1936) has suggested, the enlargement of the adrenal cortex is merely a consequence of the "profound changes in the general activity of the body as a whole" which accompanies reproductive activity.

The increase in the size of the adrenal cortex of birds and mammals at the time of follicular rupture can be related to Tuchmann-Duplessis' statement at this meeting, that the *synthetic œstrogen fenocyclin can increase the weight of the* adrenals, and also to the observations that ovariectomy in the rat leads to a transitory increase and then to the partial involution of the adrenal cortex (Bourne and Zuckerman, 1941*a*; Parkes, 1945; Burrows, 1949). In the mature male rat the reverse happens, for removal of the testes leads not to involution but to adrenal hypertrophy, an effect which can be reversed, or prevented, by means of gonadal hormones (Hall and Korenchevsky, 1938). The usual view is that this chain of responses is indirectly mediated through the pituitary, but in the light of more recent observations on hypophysectomized rats by Zizine, Simpson and Evans (1950) there is a strong possibility that androgen can directly stimulate the adrenal cortex. On the other hand, the alternative possibility that *the increase in the size of the adrenals at ovulation or* œstrus is due to the simultaneous release of gonadotrophin and ACTH is supported by the observation that in the rabbit the stimulus *of mating releases not only gonadotrophin from the pituitary* but also thyrotrophin, and possibly adrenotrophic hormone as well (Saxton and Green, 1942).

Although the adrenal cortex involutes *considerably after* removal of the pituitary, experiments on hypophysectomized rats maintained on a constant threshold dose of œstrogen suggest that it continues to fluctuate in size with the phases of the "artificial" œstrous cycles that are induced in this way (Bourne and Zuckerman, 1941*b*). If we assume that the greater part of its fluctuation during the cycle of the normal animal is due to cyclical fluctuations in the level of secretion of ACTH, it would be necessary to posit in addition the

uniformity of this kind of reaction either suggests that some types of stress may be associated with the liberation of gonadotrophin, as well as ACTH, or that the activated adrenals have a direct effect on the ovaries, a view which is not supported by the available evidence. Brolin's (1946-47) observation that exposure to cold can stimulate the secretion of both ACTH and thyrotrophic hormone favours the first possibility.

The Pathway of Motivation of the Adenohypophysis

These various observations lead me to the general conclusion that environmental changes, as defined above, may simultaneously affect several functions of the adenohypophysis. They also suggest that the effect of a particular set of conditions (e.g. deprivation of light) may have different results, depending on the physiological state of the organism. For example, a non-pregnant rat kept in the dark experiences prolonged periods of metœstrus, apparently because of a decline in FSH secretion (Fiske, 1941), whereas in the same circumstances lactation and growth become impaired in a nursing rat, due apparently to a concomitant decline in the growth-promoting and galactopoietic powers of the pituitary (Eayrs, 1951*a*). In so far as they argue against the view of a large number of distinct and specific hypophyseal reactions, and of parallel and specific pathways of stimulation, these two conclusions, if sustained, greatly simplify the problem of determining how the adenohypophysis is affected by different bodily conditions. To some extent they also simplify the question of the nature and functions of the anatomical connection between the hypothalamus and adenohypophysis, since they make it unnecessary to suppose that the hypothalamus is so differentiated that it can separately "trigger" any one of several functions of the anterior pituitary.

Harris (1948*a*, and at this meeting) has provided a careful review of the information bearing on this point, and in addition has put forward a number of observations in support of the thesis that the hypophyseal venous-portal system, which originates in a capillary network in the median eminence,

secretion of growth hormone. Pure growth hormone also has galactopoietic properties (Cotes, Crichton, Folley and Young, 1949) and it is therefore possible to inter-relate the reduced growth of the non-lactating rat kept in the dark and the poor lactating ability of the nursing female. It also follows from these observations that a normal level of secretion of both growth and galactopoietic hormone probably depends upon a normal level of light stimulation of the retina, which in turn appears to be a necessary condition of the normal growth and maturation of the gonads in rodents (Fiske, 1941). This pattern of observations thus suggests clearly that the influence of light on endocrine function may be almost co-extensive with the whole field of action of the anterior pituitary.

The Reaction of the Ovaries and Thyroid to so-called *Conditions of Stress*

The term "stress" has practically become synonymous with a very wide series of conditions (e.g. physical exhaustion, trauma, bacterial infection, exposure to extreme cold) that are commonly associated with the stimulation and subsequent exhaustion of the adrenal cortex. These adrenal changes are part of the so-called "alarm reaction" of Selye.

According to Selye (1947) the syndrome is associated in both male and female with atrophy of the gonads, "due to the shift in pituitary-hormone production which necessitates a decreased secretion of other hypophyseal principles in order to permit maximal corticotrophin elaboration." Observations made in my own department suggest, however, that both the ovaries and adrenals increase in weight in a variety of traumatic conditions (e g. surgical procedures; homografting of various tissues; application of corrosive fluid to uterine horn). In the rat, for example, both organs hypertrophy after unilateral or bilateral hysterectomy (Mandl and Zuckerman; 1951). A related observation is the finding (Mandl and Zuckerman, 1952) that breakdown of the vaginal closure-membrane occurs significantly earlier in young rats which are exposed to cold than in their undisturbed litter-mates. The

tuber cinereum adjacent to the median eminence did, Harris concludes that the way the function of the adenohypophysis is controlled is by a "chemotransmitter" which is liberated into the sinusoidal capillaries of the median eminence and "transmitted via the trunks of the portal vessels, ventrally into the sinusoids of the anterior pituitary."

Harris is undoubtedly on certain ground in emphasizing the shortcomings and equivocal nature of the evidence that has been adduced in favour of the view that the link between the hypothalamus and adenohypophysis is neural in nature. At the same time, his own thesis raises a number of far-reaching questions. Green (1951), confirming Wislocki and King (1936), points out that "the primary capillary net of the portal system does not anastomose significantly with the vessels of the neural lobe or the vessels of the hypothalamus," and Harris (1948*a*) *has also underlined the fact that the primary* capillary net is in the median eminence which, as he emphasizes, is part of the neurohypophysis. According to Ranson, Fisher and Ingram (1938), and all subsequent workers, the cellular and fibre structure of the median eminence is identical with that of other parts of the neural lobe. Harris (1948*a*) therefore talks about "the neural control of the adenohypophysis" as being "humorally transmitted from the neurohypophysis to the pars distalis," and suggests that "nervous stimuli might cause the liberation of some substance into the capillary sinusoids of the median eminence." On the other hand, he has identified his own results with those of Markee, Sawyer and Hollinshead (1946), who observed ovulation in three of four rabbits after bipolar stimulation of the "hypothalamus"—exactly where not being stated, although it is noted that the stimulation occasioned some spread, as evidenced by tremor of the head, neck and forelimbs.

The actual site of elaboration of the hypothetical "chemotransmitter" is of critical importance to the thesis which Harris supports If it is in the hypothalamus proper, as Hume, at this meeting, has also suggested, and outside the area drained by the primary capillary net of the hypophyseal

and which opens into the venous sinuses of the pars distalis of the adenohypophysis, forms a controlling link between the gland and hypothalamus. He emphasizes the unconvincing nature of the evidence that nerve fibres from the hypothalamus enter the adenohypophysis, and argues that experimental results are in favour of the view that normal anterior pituitary function persists after section of the pituitary stalk, provided the portal vessels are patent. Contrary observations he attributes to the possibility that the portal circulation had not become re-established. In experiments of his own (Harris, 1948*b*) he found that remotely-controlled unipolar electrical stimulation of the hypothalamic region of the brain caused ovulation in two of sixteen rabbits, and also in eight of a second series of seventeen animals The tip of the electrode in the two positive experiments of the first series was in the right fornix as it sweeps past the paraventricular nucleus, and in the posterior part of the tuber cinereum to the left of the midline. In the fourteen negative experiments the electrode was variously placed: in the supraopticohypophyseal tract; in different parts of the tuber cinereum; below the paraventricular nucleus; and in the neurohypophysis (other than median eminence) and adenohypophysis In the eight positive experiments of the second series the tip was "in some part of the tuber cinereum," and in the nine negative "in some part of the adenohypophysis." Harris points out that the duration of the stimulus necessary to evoke full ovulation varied considerably, and that some rabbits showed an increasing ovarian response with increasing periods of stimulation, which in the first series of experiments varied from 1 minute to 1 hour, and which in most consisted of "periods of 5 minutes applied daily for several days," and in the second series varied from 1 minute to 7½ hours.

According to Harris, the most active focus in his experiments was in the anterior wall of the tuber cinereum just above the anterior part of the median eminence. Since direct stimulation of the adenohypophysis and of the infundibular stem failed to induce ovulation, whereas that of the part of the

transmitter produced in the median eminence may merely be some substance which controls vasodilatation or vasoconstriction in the pars distalis, the implication being that some such process alone may be responsible for a particular pattern of pituitary secretion. Harris (1948*a*), as well as Green (1951) also discusses the possibility that the chemotransmitter is an adrenaline-like substance. Green, on the other hand, has underlined the highly conflicting nature of the evidence that concerns the "blocking" of coitus-induced ovulation in rabbits by means of various drugs, and the stimulation of ovulation by injecting adrenaline directly into the pars distalis (e.g. Taubenhaus and Soskin, 1941; Sawyer, Markee and Hollinshead, 1947; Sawyer, Markee and Everett, 1950), and has concluded that it is "too soon to concede that either an adrenergic or cholinergic link exists" between the adenohypophysis and hypothalamus.

A final and more general issue, that is brought to the fore by these various possibilities, affects general ideas about the nature of humoral action As originally set out by Bayliss and Starling in 1905, and as elaborated since then, the basic endocrinological concept is that there are tissues which are specialized to produce and liberate into the systemic blood stream specific chemical substances which in the course of their circulation round the body affect the function of certain target organs. The concept does not presuppose any specific anatomical connection between the endocrine organ and the tissues which it influences, thus androgen produced in the testis stimulates the cells of the comb and wattles of the head of a rooster. Its critical feature is that of the interaction between a specific chemical stimulant and a specific substrate. The notion of chemotransmitters which influence the action of the adenohypophysis falls quite readily into this general scheme, in the same sense as does the knowledge that the adrenal cortex is controlled by a specific secretion of the anterior pituitary. From this point of view it neither matters that the nature of the possible chemotransmitters of the median eminence is unknown, nor that we do not know by

portal vessels (i.e. the median eminence), then it is difficult to see why the presumed "humoral transmitters" should not be swept into the systemic circulation—unless it were to be argued, as could be from the observations of Bargmann (1949) and Bargmann and Scharrer (1951), that they pass within nerve sheaths from hypothalamic neurones into the median eminence. If the latter view is advanced, it would be necessary both to explain how the tractus hypophysius is so specialized for the molecules in question to be distributed within the median eminence only, and not throughout the neurohypophysis, and to dispose of the convincing thesis that the neurosecretory material of the hypothalamus passes by "axonal transport" into, and is identical with, the protein hormone of the pars nervosa (see, for example, Smith, 1951). If, on the other hand, the chemotransmitters are produced in the median eminence itself, the zone of the primary capillary net, which is what is suggested by some of Harris' descriptions, it becomes necessary to explain, first, how the hypothalamic impulses which provoked ovulation both in his own and Markee's experiments, reached the median eminence, and second, why, in spite of its uniform histological structure, only part of the neurohypophysis is specialized as a controlling centre of the adenohypophysis (e.g. Harris notes that stimulation of the infundibular process and stem had negative results).

Another important issue which is raised concerns the possible specificity of the chemotransmitters of the median eminence. Unless it be assumed that the adenohypophysis secretes a balanced pattern of its hormones when subjected to stimulation by some single humoral substance "produced" in this part of the neurohypophysis, or in the adjacent part of the hypothalamus, the pattern altering according to the strength of the stimulus, it follows that a number of different chemotransmitters can be released in different circumstances, each being responsible for some specific pattern of secretion by the pars distalis.

Harris has referred, but only briefly, to this consideration. He mentions a suggestion of Green (1951), that the chemo-

in a corresponding way. Another is the stimulation of gonadal activity in anœstrous ferrets by means of light. This problem has been under investigation over the past three years in my own laboratory, but for a number of "technical hitches," ferret distemper not least among them, no results worth reporting have so far emerged—except the observation that blind ferrets, contrary to published reports, frequently come into season at the same time of the year as do normal ones. The observations which Harris and Jacobsohn have reported at the present meeting would lead us to expect that the results of the experiment we are carrying out will be negative except in the case of sellar grafts in which a portal circulation has re-established itself.

Whatever their nature, and however useful it will be to establish one way or the other whether the functioning of the adenohypophysis is dependent, as Harris postulates, on the integrity of the portal vessels, it is essential to realize that such experiments will not answer the greater part of the problem of how the pituitary responds to environmental changes. The central issue of the problem is the translation of a complex and continually varying pattern of hormonal stimulation. Such hormonal links as have been posited, for example, adrenaline, which according to Sawyer *et al*, 1950, may stimulate the release not only of ACTH, but also of thyrotrophin, impart to the problem too simple a character. So, too, does the emphasis on isolated anatomical links We shall only end by deluding ourselves if we fail to remember that the problem of the way in which the pituitary reacts to environmental stimulation is at least as obscure as the way in which the hypothalamus modulates the functions of the body, and so helps to integrate them in patterns of adaptive responses.

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what hypothalamic cells they are produced—whether they be the cells of the median eminence, or those of the nuclei supraopticus or paraventricularis, as suggested by the observations of Bargmann and Scharrer. What is important is that the notion of such chemotransmitters is bound up with the idea that they are conveyed along a specific anatomical pathway. What is also important is that the evidence now gathering leads clearly to the conclusion that the neurosecretory substance of the hypothalamus is released and stored in the neurohypophysis. These two issues introduce a completely new aspect into general discussions of the specificity of cellular reactions of the kind with which we are dealing. They cannot be properly evaluated until we are certain that the anatomical integrity of the pituitary-hypothalamic connection is essential, as a general rule, for the functioning of the adenohypophysis, and that the function of this part of the pituitary is in some way controlled by the neurohypophysis.

The papers that have already been given at this meeting have indicated many areas in which facts as well as interpretations are conflicting and to that extent at least, unconvincing. The critical proof that the anatomical integrity of the portal circulation is essential for the normal functioning of the anterior pituitary, or for that matter, any other anatomical link (e.g. neural) between the hypothalamus and pars distalis, would be the demonstration that pituitary grafts do not maintain normal bodily function, or alternatively that normal function is impossible after the pituitary is separated from the hypothalamus. The literature on this subject which Harris (1948*a*) reviews is equivocal. More recently, Cheng, Sayers, Goodman and Swinyard (1949*a*, *b*) have shown that pituitary grafts in the anterior chamber of the eye will release ACTH, as shown by the ascorbic acid test, following the injection of histamine. This observation has been confirmed at the present meeting by Fortier, and also in an earlier paper by himself and Selye (1949). This is only one of many specific hypophyseal responses that needs to be tested

STUDIES WITH RADIOACTIVELY LABELLED ANTERIOR PITUITARY PREPARATIONS*

MARTIN SONENBERG†

THERE is now a considerable body of evidence which suggests that proteins may be labelled with substituent groups and retain to a considerable degree their biological properties. Both with *in vitro* as well as with *in vivo* techniques the bio- [illegible] to those of [illegible] generally [illegible] ent groups. For example, various groups have been coupled by the diazo linkage to antigens (Landsteiner *et al.*, 1917, 1918) and their reactivity with antisera studied. R-salt-azo-benzidene-azo-egg albumin was prepared (Heidelberger, Kendall and Soo Hoo, 1933) as an antigen and this permitted the quantitative colorimetric determination of antigens. Labelled anti-typhoid serum (Marrack, 1934) has been prepared by coupling "R" salt to one end of bisdiazotized benzidene and anti-typhoid serum to the other. Fluorophores coupled to antiserum (Coons *et al.*, 1941, 1942) have been used to trace the coupled antiserum in tissues by ultraviolet light.

More recently, radioiodinated antigens have been prepared to study the immunological reactivity of bovine serum albumin (Eisen and Keston, 1949). These preparations have been found in quantitative precipitin studies to react in a manner indistinguishable from unlabelled serum albumin. When antibodies have been labelled with ^{131}I, there has been no loss

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the preparation gives no indication of the biological activity of the labelled molecules, since the bulk of the molecules are essentially the unreacted biologically active molecules. The interpretation of any experiment involving the labelling of a protein preparation is conditioned by the degree of homogeneity of that preparation. The rates of labelling of each constituent in a non-homogeneous preparation may vary widely (Pressman and Sternberger, 1950). The average number of substituent groups per protein molecule in a heterogeneous preparation may not therefore reflect the average number of substituent groups attached to the biologically active constituent.

With these possibilities and limitations in mind we have studied the biological activity of several labelled anterior pituitary preparations. In these studies hormone preparations have been labelled by iodination with ^{131}I or by coupling the protein preparation with ^{35}S diazobenzenesulphonic acid. The non-protein bound radioactivity was dialysed free of the labelled protein preparation. The latter was used for determination of the biological activity as well as for localization studies.

It has been found (Li, Lyons, Simpson and Evans, 1940) that prolactin preparations iodinated with an iodine to protein molecular ratio of 12 to 1 still retained some biological activity. If substitution to this extent did not destroy all the biological activity, one might expect greater retention of activity with trace labelling. Prolactin preparations have been labelled with iodine containing ^{131}I until there was an average of 1·6 iodine atoms substituted for each protein molecule. For these determinations, we have assumed a homogeneous protein of molecular weight of 30,000 (White, 1949). Such a preparation has been found to retain all its biological activity as assayed by the pigeon crop sac technique (Sonenberg, Money, Keston, Fitzgerald and Godwin, in press) It has also been reported (Cox, 1951) that there has been retention of biological activity of prolactin after labelling with ^{131}I and obtaining an average of 2 iodine atoms per

in their ability to precipitate specific antigens. When inoculated into rats ^{131}I labelled anti-tissue antibodies have localized at the specific tissue against which they were prepared (Pressman *et al.*, 1948, 1949*a*, 1949*b*, 1949*c*, 1950*a*, 1950*b*, 1950*c*, 1950*d*; Eisen and Pressman, 1950; Eisen, Sherman and Pressman, 1950; Masouredis, Melcher and Koblick, 1951). ^{35}S diazobenzenesulphonic acid has also been used to label antibodies and study their *in vivo* localization (Pressman *et al.*, 1950*e*).

New biological properties in addition to the old ones have been reported after such procedures as the coupling of a diazonium compound to protein or the direct iodination of protein. The average number of groups substituted on the protein molecule and the amino-acid substituted will determine either the loss of some biological property or the acquisition of another. After insulin was coupled (Reiner and Lang, 1941) with 6 azo groups per protein molecule it was as active biologically as an unlabelled preparation. Insulin coupled with diazotized *p*-iodoaniline with radioiodine has been used to study the absorption and distribution of insulin (Reiner, Keston and Green, 1942; Reiner, Lang, Irvine, Peacock and Evans, 1943; Root, Irvine, Evans, Reiner and Carpenter, 1944). Labelled insulin has been prepared by the direct iodination of the protein hormone with ^{131}I (Ferrebee, Johnson, Mithoefer and Gardella, 1951).

The reaction of iodine with a protein may not only lead to substitution on the protein molecule but may also result in oxidation or other reactions which may additionally alter the biological activity of the protein. It has been reported (Herriott, 1947) that largely substitution without oxidation took place rapidly above *p*H 5. When a protein preparation is labelled effectively with reagents in amounts smaller than one reagent molecule per protein molecule, as is possible with radioactive substances, the probability that the same protein molecule will be both labelled and undergo any other chemical reaction, e.g. oxidation, is small. Although such preparations may be most useful in tracer experiments, biological assay of

I ... site of binding of an active labelled

may be compared with other radioactive substances without the same hormonal activity. Presumably there should be

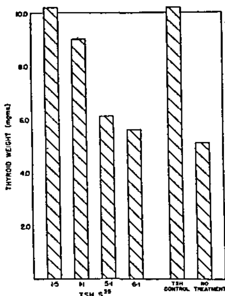


FIG 1. Biological activity as measured by chick thyroid weight of various thyrotrophic preparations. The ratios of the TSH ^{35}S preparations refer to the average ratios of sulphonic acid group to protein TSH control refers to a group of chicks receiving an unlabelled thyrotrophic preparation.

greater localization of radioactivity in the target organs of the labelled specific material. Thus, there is greater concentration of radioactivity in the rat adrenal after injecting a ^{131}I labelled ACTH preparation than with either inorganic iodide labelled with ^{131}I or a radioiodinated bovine serum albumin preparation (Sonenberg, Keston and Money, 1951). When a radioiodinated growth hormone preparation was administered,

protein molecule. Preparations with adrenocorticotrophic activity labelled with ^{131}I have also been found to retain their biological activity (Sonenberg, Keston and Money, 1950*a*, 1951; Ferrebee, Johnson, Mithoefer, and Gardella, 1951). Such hormone preparations retained considerable activity after treatment with several non-radioactive iodine atoms per molecule of protein (Li, Simpson and Evans, 1946). Protein preparations with thyrotrophic activity have been labelled by coupling the protein to diazotized benzenesulphonic acid labelled with ^{35}S. For this purpose carrier-free ^{35}S sulphanilic acid was prepared (unpublished experiments). When labelled with a sulphonic acid to protein average molecular ratio of 1 to 6, there was no loss of biological activity. Complete loss of biological activity was noted when the average molecular ratio of sulphonic acid group to protein reached 6 to 1 (Fig. 1). For these calculations it was assumed that we had a homogeneous protein of molecular weight of 10,000 (White, 1944). Since many of these preparations were non-homogeneous, such information must be interpreted with considerable reserve.

On theoretical grounds, a hormone may be considered first to be bound to its end organ and then to activate this gland. These two properties of the hormone may be independent of each other. The ability to activate may conceivably be lost with retention of ability to bind with its end organ. This may be similar to the case of diphtheria toxin, where treatment with formaldehyde (Eaton, 1937) destroys toxicity with retention of antigenicity. In the case of our labelled prolactin and ACTH preparations, however, it is likely that both the biological activity and the localizing potential have been retained. In our tracer experiments with labelled anterior pituitary preparations, we have attempted to label these preparations in such a manner as to retain their biological activity. This has been approached by labelling with either traces of labelling reagent or with amounts of iodine which were demonstrated not to destroy appreciable biological activity.

anterior pituitary preparation. Preparations with known biological effects on one organ may show greater localization of radioactivity in that organ than in another which is not known *to respond to the trophic preparation. For example, there* was a greater degree of localization of radioactivity in the adrenal than in muscle after the administration of a ^{131}I labelled ACTH preparation (Sonenberg, Keston and Money, 1951). This approach should not preclude, *a priori*, the localization of radioactivity at an unexpected site.

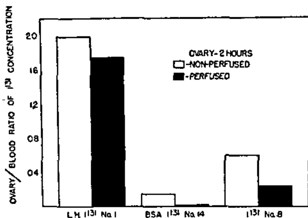


FIG. 3. Effect of perfusion on the localization of radioactivity in the ovary two hours after the administration of ^{131}I labelled substances.

Fourthly, it has been noticed that radioactivity which has localized in an organ is to a greater extent retained in that organ after perfusion when the material administered apparently *has a specific relationship for that organ. In the case of* a ^{131}I labelled prolactin preparation (Sonenberg, Money, Keston, Fitzgerald and Godwin, in press) or a ^{131}I labelled luteinizing hormone preparation (Fig. 3) less than 15 per cent of the radioactivity in the ovaries was lost by perfusion. There was a greater loss of radioactivity from the ovaries by perfusion after the administration of radioiodinated bovine serum albumin or ^{131}I labelled inorganic iodide.

there was greater localization of radioactivity in the pancreas than when ^{131}I labelled preparations rich in other pituitary fractions were given (Fig. 2). This was also greater than the concentration of radioactivity with radioiodinated bovine serum albumin or inorganic iodide labelled with ^{131}I.

Secondly, it may not be sufficient to make actual comparisons between the concentration of radioactivity between experimental and control substances. The levels of radio-

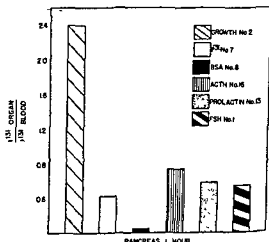


FIG 2. Localization of radioactivity in the pancreas one hour after the administration of ^{131}I labelled substances.

activity in the blood may determine to some extent the amount of radioactivity in an organ. To obviate this, the concentration of radioactivity in an organ may be compared with that in blood at the same time. Such a comparison will reveal an absolute concentration in an organ which could not be accounted for by the blood content alone of that organ (Figs. 2 and 3). This may suggest either binding or penetration into cells by one or more components of the labelled preparation.

Thirdly, comparisons may be made between different organs of the same animal receiving a radioactively labelled

component either in perfusion or in tissue fixation. The absence of significant concentration of radioactivity may, however, really exclude the mammary tissue as a site of action of prolactin preparations. Early experiments with labelled adrenocorticotrophic preparations suggested the kidney as a site of localization for the pituitary factor. But as more active preparations became available less of the administered material localized in the kidney (Fig. 4).

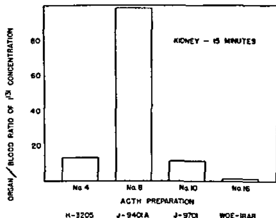


FIG 4 The localization of radioactivity in the kidney 15 minutes after the administration of ^{131}I labelled ACTH preparations Each animal received 100 micrograms of protein The potency with reference to the LA-1-A standard was.—

H—3205 —2 5
J—9401A—1 8
J—9701 —1 7
WOE—18 AR—100

The data obtained from the degree of localization of radioactivity in organs at different times after the administration of radioactively labelled hormone preparations may be employed to estimate the possible turnover rate of these substances. Such interpretations must be made with the realization that we are measuring not only the labelled material of a possible specific nature but also labelled contaminants and the metabolic products of all these substances, which may

Fifthly, when comparisons were made between two ^{131}I labelled ACTH preparations with different adrenocorticotrophic activity, as measured by adrenal ascorbic acid depletion, there was a difference in the degree of localization of radioactivity (Sonenberg, Keston and Money, 1950*b*). After perfusion, more radioactivity was retained in the adrenal after the injection of the labelled ACTH preparation with greater adrenocorticotrophic activity.

Finally, autoradiography may be employed to define more precisely the histological area of the localized radioactivity. The radioactivity in the ovary after the administration of a ^{131}I labelled prolactin preparation seemed to predominate in the corpora lutea, using the autoradiographic technique (Sonenberg, Money, Keston, Fitzgerald and Godwin, in press). Of the radioactivity which has localized in the kidney after the administration of a ^{131}I labelled growth hormone preparation the bulk was in the convoluted tubules. After the administration of all our labelled protein hormone preparations, the radioactivity in the kidney has shown a preference for the convoluted tubules.

The use of labelled anterior pituitary preparations may fail to suggest a site of action of a particular anterior pituitary factor. When a ^{131}I labelled prolactin preparation was administered to female rats there was no significant localization of radioactivity in mammary tissue (Sonenberg, Money, Keston, Fitzgerald and Godwin, in press). Pregnancy or lactation in rats did not seem to alter the pattern of radioactivity localization. This might at first appear disconcerting, but several explanations for this observation are possible. In a non-homogeneous hormone preparation, the factor acting on mammary tissue may represent a small fraction of the extract used. The specific factor would then be limited in its degree of localization. The inclusion of non-mammary tissue with mammary tissue in our radioactivity assay would also result in a low concentration of localized radioactivity. Negative autoradiographs of mammary tissue may merely reflect a loose binding which would permit the loss of a specific

after being placed in the cold for four hours, have demonstrated a greater concentration of ^{131}I in the adrenal than normal animals after the administration of a ^{131}I labelled ACTH preparation (Fig. 6). The differences noted were not as marked between the chilled and normal animals when injected with radioiodinated bovine serum albumin or ^{131}I alone. Whether this is a result of stress, increased circulation through adrenals, or other reasons, we cannot say at present.

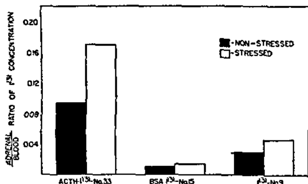


FIG. 6. The localization of radioactivity in the adrenal one minute after the administration of various ^{131}I labelled sub-

In conclusion, it would appear that anterior pituitary preparations may be labelled with various radioactive reagents. Such tagged preparations may then be employed to study the behaviour of the anterior pituitary factors under varying physiological conditions. Caution should be exercised in the interpretation of the data obtained with these techniques. The non-homogeneity of pituitary preparations, the inability to precisely characterize the localized components, biological variations and other unknown variables emphasize the need for qualified conclusions. Further study is in progress in this laboratory to explore the usefulness and limitations of this method.

be retained in the organ. *Such retention may prolong the apparent biological half life.* Notwithstanding, *it is interesting to note that the biological half life of radioactive components* in the adrenal from a labelled ACTH preparation (Sonenberg, Keston, and Money, 1951) was similar to results obtained by biological assay of plasma (Greenspan, Li and Evans, 1950). There may be different turnover times for endogenous and exogenous ACTH, especially when the latter

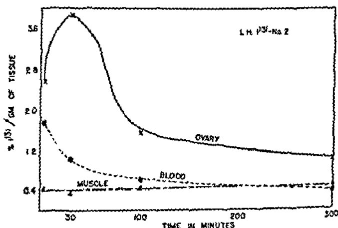


FIG. 5. The localization of radioactivity in various tissues after the *administration of a ^{131}I labelled luteinizing hormone* (L.H.) preparation.

has been administered in excessive amounts. The half time *of turnover of localized components* in the adrenal after administering labelled ACTH preparations was about 5·5 *minutes*. In the ovary, *the turnover rate of localized radio*activity after injecting radioactively labelled prolactin preparation *was approximately 90 minutes* (Sonenberg, Money, Keston, Fitzgerald and Godwin, in press), and after *injecting labelled luteinizing hormone preparations* was about 85 minutes (Fig. 5).

An attempt has been made to employ labelled anterior pituitary preparations in a more dynamic fashion. Rats,

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DISCUSSION

Folley: It seems to me that the mammary intra-duct injection experiments of Lyons, carried out on rabbits, in which he injected minute amounts of purified prolactin direct into individual galactophores of the rabbit mammary gland, show definitely that the hormone does have a direct effect on the epithelium, and I don't at the moment see how his results can be reconciled with your findings.

I might add that I have since noticed a short abstract in the *American Journal of Anatomy* by Dr. Cox in Montreal, in which she states she detected the radioactivity in mammary tissue and milk. That was in mice. Our work was in rats.

Pochin: Presumably your data on mammary gland could be explained if prolactin was concentrated there but happened to have a rapid turnover.

Sonenberg. Presumably that's true, but in the case of the ACTH preparation, which seems to have the most rapid turnover—it has a biological half-life of about 5.5 minutes in the adrenal gland—we were able to detect it. It would have to be even more rapid than that.

Acknowledgements

Dr. William L. Money and Dr. Albert S. Keston were active col-
laborators in this project. I should like to
to Dr. Rulon W
C. Peacock w
Miss Jean F
Eva Simmel ar
help in this pro

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J.

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they were iodinated with only small amounts of iodine, in contrast to some of your experiments. (By "small amounts," I mean we would introduce less than two groups per protein molecule if we had a homo-

the activity of growth hormone, ACTH, and lactogenic hormone was abolished on iodination We used very extensive iodination; but perhaps in your case only one or two groups of tyrosine in the molecule are iodinated.

Sonenberg: No, we have just assumed an average molecular weight for the protein and then determined the amount of iodine in the protein

that in a matter of five hours all the iodine went in the thyroid. Your experiments require only a matter of minutes, and it is quite possible that in such a short time no iodine goes to the thyroid. I wonder whether by using thyroidectomized animals more iodine would be localized in the glands you want to study.

Courrier: Are you sure that in your experiment the radioactivity remains bound to the hormone?

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some specificity for different tissues, even if the biological activity isn't retained. We certainly haven't resolved that to our own satisfaction, that the biologically active molecules are also the labelled molecules.

Morris: Couldn't the problem of whether the radioactivity is still

attacked by

ways, rather

the iodine

attached in

some other way. The two groups would have to be split off at the same rate in order to maintain a constant ratio in your localized site. It increases the probability.

Sonenberg Yes, that would be a good suggestion.

YOUNG: Was your labelled growth hormone fully active biologically?

SONENBERG. The growth hormone has not yet been assayed for

as yet.

NELSON: I wonder if Dr. Sonenberg would like to tell us where prolactin is localized in the male. The action of prolactin in males has been a source of much concern to those of us who are interested in male reproductive physiology.

SONENBERG: With prolactin in the male, we've only done experiments with 20 animals. If you want to accept it in the light of these small numbers, we've noticed it in the prostate in males, and in these 20 animals it's been somewhat consistent.

NELSON· Did you detect which part of the prostate?

SONENBERG: We found it in both the ventral and dorsal prostate in the male rats. I have no idea what that means.

LI: I was wondering about the purity of the lactogenic hormone, because in our experience the ICSH (LH) content of lactogenic hormone

enough, we did have localization of radioactivity in the adrenals after administrating these radioactive prolactin preparations.

NELSON· I think the preparation you used was one that Dr. Bates made in very large amount He assured me it was free of gonado-

effects of œstrogen. However, when we used hypophysectomized animals, we found that it did indeed have an ICSH action. So I think at least in the male this particular preparation of prolactin must be

you did not get loss

by iodination in an

arbonate buffer and

*The labelled growth hormone preparations have since been found to be biologically active

ATTEMPTS AT INHIBITION OF ANTERIOR LOBE SECRETION BY *p*-HYDROXYPROPIOPHENONE

A. LACASSAGNE

DURING the last two years, various papers have drawn attention to the physiological effects and therapeutical possibilities of *para*-hydroxypropiophenone (PHP), a chemical which had been known for more than 60 years, but whose biological activity had not been investigated. Therefore a brief historical survey seems suitable.

History

Since January 1944, when my co-workers and I put forward a first example of competitive antagonism in the carcinogenic action of two polycyclic hydrocarbons with close configurations but different activities, an extensive team-work has been undertaken in order to prepare and test a number of chemicals considered as possible inhibitors, either as "anti-carcinogens" or "anti-œstrogens."

For this last study, our organic chemists prepared a series of various compounds, the œstrogenic activity of which was determined according to the Allen-Doisy test; the results of these first experiments were published in 1946 (Corre *et al.*). Among the tested substances were PHP, which was inactive at a dose of 5 mg., and its succinate, weakly œstrogenic.

Meunier and his co-workers (1943) had previously observed an antagonism between dicoumarol and half of its molecule: 3-methyl-4-hydroxycoumarin. In the same way, one might suppose that stilbœstrol (I) could be antagonized by PHP, half of its ketonized molecule (II)

As the synthesis of PHP previously described by Perkin (1889) and even that of Goldzweig and Kaiser (1891) gave poor yields, a new type of synthesis was worked out by my

LI: In your experiment, when this radioactive iodine goes to the adrenal, in a matter of two to three minutes after injection of iodinated ACTH, you found that the higher the activity of the material the more material migrates to the adrenal, and the converse. Could that be used as an assay method?

SONENBERG: Well, it *could*, if there was direct correlation between the degree of localization and the purity.

LI: I meant for an assay of activity rather than purity; the purity and activity might not go hand in hand.

It can be concluded from Table I that PHP is about 35,000 times less œstrogenic than stilbœstrol, when given subcutaneously, and 10 times less when given orally.

Action on the Growth of Young Animals. Several litters of mice and many young rats received a diet containing 5 to 10 mg. of PHP per day. In general the growth was slowed down or even stopped. The treatment could be maintained, without other trouble, for more than a year, but the female remained sterile.

Table I

Mode of administration	*Dose in mg*	*Per cent of positive œstrus*
SUB-CUTANEOUS (total dose after 5 injections in 36 hours)	10 12 15	50 80 90 to 100
ORAL (total dose ingested in 36 hours with 10 to 12 g of food)	10 to 50 100 120	0 20 90

Table II is an example of an experiment started on 4 Wistar 6-week-old rats, to which a total dose of 3·6 g. PHP was given orally in 30 days.

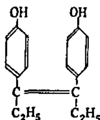
Table II

	Controls	*Treated*
Number of animals	2	2
[illegible]	86	93
[illegible]	217	141
[illegible]		
[illegible]	4 3	4·8
Thyroids	10·1	10 6
Adrenals	16 1	31·7
Testis	1182	395
Seminal vesicles + prostate	260	33
Sub-maxillary glands	161	161

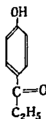
A striking discrepancy appears between the inhibition of growth of the gonad (weight 33 per cent of the control) and of the accessory sexual glands (weight decreased by 89 per cent)

co-worker Buu-Hoi (1949) which made large quantities of this substance available to the experimenters. Meanwhile, Dr. Perrault to whom PHP had been given for tests at the Hopital Lariboisière, obtained with it a surprising therapeutical result: the disappearance of multiple lung metastases in a case of generalized chorio-epithelioma (Perrault *et al.*, 1949).

The publication of this case aroused curiosity about the biological properties of PHP from both physiological and therapeutical standpoints. Our first results in mice and rats have been recently published (Lacassagne *et al.*, 1950). The



(I)



(II)

present status of our research in this field can be summarized in the following way.

Results Obtained in Our Experiments

Mode of Administration. PHP is practically insoluble in water and in oil. Heated in a water bath for 15 minutes at 100° in physiological saline, it gives a stable solution of 1 mg. per ml. The best solvent is propylene-glycol, which can dissolve 40 mg. per ml. at 52°.

Such preparations have been used in sub-cutaneous injections mainly for a study of the œstrogenic potency. For long treatments, the drug was mixed with the food and given orally.

Œstrogenic potency was established with accuracy in ovariectomized rats with both modes of administration.

Anti-goitrogenic Action in the Adult Rat. But the fact that PHP slows down the goitrogenic action of propylthiouracil can be proved by Chamorro's test (1949).

Table IV includes: (*a*) 6 controls; (*b*) 6 animals which received by mouth 120 mg. of PHP daily during 24 days and which, from the 11th day on, were given 1 injection a day of 1 mg. propylthiouracil (a 10^{-3} solution at *p*H 7·4), during 14 days; (*c*) 6 animals which were given this second treatment only.

Table IV

Number of animals	*Treatment*	*Total weight of the thyroids in mg*	*Weight of the thyroids in mg per 100 g animal weight*
6	Controls	18 1	8 4
6	PHP+propylthiouracil .	20 6	9 6
6	Propylthiouracil alone . .	33 7	15 7

From this table, hypertrophy of the gland due to propylthiouracil acting by exciting the secretion of thyrotrophic hormone, is mostly inhibited by PHP.

Interpretation of Our Results. Besides the experiment which has just been reported and which shows an inhibiting action of PHP on the secretion of the thyrotrophic hormone of the pituitary, it may be concluded from the results obtained in male rats that there is a marked and early inhibition in the production of LH and perhaps a delayed one of FSH. In fact, despite the weak œstrogenic power of this substance, a marked atrophy of the accessory sexual glands is followed by the regression of the seminal line, the activity of which is not completely suppressed, even in growing animals.

In contrast with this depressing influence on certain secretions of the anterior lobe, is the absence of macroscopic and microscopic changes in this gland after PHP, whereas œstrogens, given over a long period to mice and rats, lead to hyperplasia, very important sometimes, ending with the formation of adenomas.

on the one hand, and the lack of effect on the thyroid and the hypertrophy of the adrenals (about 100 per cent) on the other hand. Histologically, the testis alone displays important changes· the cells of the seminal line (which is cut before sperm is formed) show many abnormalities with aspects of cytolysis. These important changes in the sexual glands are obtained with a dose which, from the œstrogenic potency standpoint, corresponds to the absorption of one rat-unit per day, in the case of the oral administration of 110–120 mg. of PHP.

Action on the Sexual Organs of the Male Adult Rat. Daily doses of PHP given for a long time to adult mice and rats cause progressive atrophy of the testis and of the accessory glands. The effect on the gonad appears slowly, the atrophy being marked after 4 months. Atrophy appears much earlier in the seminal vesicles and the prostate.

Table III is an example of an experiment carried out on 4 Wistar adult rats which were given a total dose of 3·6 g. PHP in 30 days

Table III

	Controls	*Treated*
Number of animals	2	2
Average weight in g.	316	311
Average weight after 30 days	312	297
Average weight of the organs in mg:		
Pituitary gland	10 1	8 9
Thyroids	20 6	17·7
Adrenals	43	35·3
Testis	2862	2906
Seminal vesicles + prostate	1548	1120
Sub-maxillary glands	413	350

The marked regression of the seminal vesicles, of the prostate (28 per cent) and of the sub-maxillary gland (15 per cent) after treatment, is significant; it contrasts with the lack of effect, after one month, on the weight of the testis, in which histological examination fails to detect any injury in the seminal line. The experiment also does not show any evident effect on the thyroid.

Therapeutical Effects. Observations made by physicians agree with those of the experimenters. We shall not speak here of the few happy regressions of malignant tumours (to which a much larger number of failures should be opposed); this is a different problem, which is out of place in this talk. But numerous observations have been published, reporting improvements after PHP in von Basedow's disease and in hyperfolliculinæmia where the hormonal equilibrium was re-established (Perrault, 1950; Ezes, 1951). The mechanism of this action seems to consist in the slowing down of a hyperproduction of thyrotrophic and gonadotrophic hormones.

Conclusions

It is still too early to explain how PHP, a very weak œstrogen, intervenes in slowing down the general growth of young animals, in reducing the volume of the accessory sexual glands (seminal vesicles and prostate), in producing the atrophy of the testis, and in antagonizing the goitrogenic action of propylthiouracil, but one can suggest that such actions are due to an inhibition of certain secretions of the anterior lobe of the pituitary gland.

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Results Obtained by Other Experimenters

The results of Soulairac and Desclaux (1950) lead to conclusions which fit in with the preceding ones. With doses from 60 to 560 mg. of a solution of PHP propionate in oil, injected subcutaneously to rats, these authors obtained a progressive involution in the epithelium of the glands of the uterine horns and a decrease in thickness in the epithelium of the seminal vesicles, of the prostate, and mainly of the vas deferens, with the disappearance of the phosphatase activity. Moreover, they noticed (1951) a few histological changes in the pituitary gland, i.e. some vacuolization of basophil cells.

Martella (1951) noticed some alterations in the œstrous cycle of rats receiving 25 cg. of PHP per day in their food; he attributes them to an anti-gonadotrophic action which exerts itself more probably on the pituitary gland than directly on the ovaries.

Loeper and his co-workers (1951), injecting PHP to rats, have prevented the hypertrophy of thyroid and pituitary glands after treatment with propylthiouracil. But, on account of the very high doses of this substance they have used, they could not prevent histological lesions in these glands.

On the other hand, Guillemin (1951) got results which prevented him from admitting an action on the growth of young male rats. With daily doses of 10, 30 or 50 mg. of PHP given orally during 18 days to animals weighing 80 g. (the doses being doubled during the last 8 days), he did not observe any statistically significant difference between the growth of the treated animals and of the controls. At the end of the experiment, no macroscopic or microscopic alteration of the gonads, thyroid, adrenals, thymus, or kidneys, could be found.

How can we understand such a complete discrepancy? First, it seems that the doses given by the last author were too small, and that the treatment was performed during too short a time. Secondly, we used for a while some commercial PHP which had not been synthesized in our laboratory by Buu-Hoï's method; this substance proved totally inactive, even with higher doses and longer treatment.

Therapeutical Effects. Observations made by physicians agree with those of the experimenters. We shall not speak here of the few happy regressions of malignant tumours (to which a much larger number of failures should be opposed); this is a different problem, which is out of place in this talk. But numerous observations have been published, reporting improvements after PHP in von Basedow's disease and in hyperfolliculinæmia where the hormonal equilibrium was re-established (Perrault, 1950; Ezes, 1951). The mechanism of this action seems to consist in the slowing down of a hyper-production of thyrotrophic and gonadotrophic hormones.

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before we instituted any form of therapy, and we had observed on three occasions that her pick-up of radioactive iodine in the thyroid was in the neighbourhood of 70–75 per cent. After six weeks of treatment with the *p*-hydroxypropiophenone there had been no change observed in her eyes. However, the iodine pick-up had fallen off to 20 per cent, which I think is rather a significant change.

Lacassagne: Dr. Cowie has shown negative experiments, but it seems to me that a duration of 17 days is not enough In a paper by Guillemin of Montreal, published a few weeks ago in the *Presse Médicale*,

Dr. Rawson has clinical observations We have not done experiments on patients, but Dr. Perrault in the Hôpital Lariboisière has done

may be due to different reasons Perhaps the doses were inadequate, these doses were believed to be adequate at the beginning, but now we have seen that Dr Lacassagne is using very high doses.

Another possibility is the duration of the experiment. Or perhaps it is an inactive preparation. He has used a preparation of Laroche Navarron In these problems the positive results with a pure preparation are more important than negative, and Dr. Lacassagne has shown us very important positive results Another possibility could be that some very active contaminant might be present in some preparations and not in others I mentioned the negative results because we have to see what is the reason for them

Gross. Dr. Schuler did some experiments in our laboratories with the preparation of Laroche Navarron and found a decrease of gonadotrophic hormone in pituitaries of rats treated with 100 mg./kg. orally daily for 10 days.

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DISCUSSION

COWIE Recently at Shinfield rumours reached us of this interesting substance and it seemed that it would be of interest to find out if it would inhibit those secretions of the anterior pituitary which are essential for lactation. Up to now our experiments have given negative results We measured the growth rate of the litters of the three following groups of lactating rats (1) a control group; (2) a group in which each rat was implanted with a 50 mg. pellet of *p*-hydroxypropiophenone every four or five days (mean daily absorption = 9 25 mg.); and (3) a group which received daily subcutaneous injections of a supersaturated solution of this substance in saline (12 5 mg /day) There was no evidence of inhibition of growth of any of the litters of the treated rats, showing that *p*-hydroxypropiophenone had no inhibitory effects on milk secretion These treatments were carried out from the 4th to the 17th day of lactation, after which time the mothers were killed and the endocrine glands and the mammæ examined histologically. So far I have had the opportunity to examine only a few slides, but in these I have observed no differences between the tissues from treated and control animals Sections from the mammæ, the ovary, and the adrenal gland were all normal While these results give no indication of any inhibition of the galactopoietic or other factors of the anterior pituitary, it must be stressed that these rats were treated only for a total of 13 days, which may have been too short a time for the effects of *p*-hydroxypropiophenone to show up

RAWSON. We have been doing some studies with *p*-hydroxypropiophenone in rats, and in one human. We have administered *p*-hydroxypropiophenone to rats receiving thiouracil or to rats on iodine-deficient diets and have followed the effects of the agent on the thyroid weight and on the capacity of the thyroids to concentrate or pick up radioactive iodine With doses of 10, 25, 50 and 100 mg daily, we have observed no effect of the *p*-hydroxypropiophenone on the thyroids of these animals.

One thing that is important in comparing these experiments is that Dr Lacassagne worked with the Wistar rat, whereas we have been using the Sprague-Dawley rat I wouldn't have believed that that could have made much difference, excepting for this fact. We have been studying iodine metabolism in the Sprague-Dawley rat on a Remington diet for a long period of time and have been able to get maximum pick-up of radioactive iodine. Other investigators in the United States have been unable to get these same effects if they were using the Wistar rat. However, if they changed to the Sprague-Dawley rat they did get this difference in pick-up. There may be some difference in the thyroid metabolism in the two animals.

PART III

GROWTH HORMONE

THE GROWTH HORMONE AND CARBOHYDRATE METABOLISM

F. G. YOUNG

In a conference on "Control of the anterior pituitary and reciprocal relationships between its secretions and those of the target organs" a paper concerning the growth hormone may seem to be not entirely relevant since, as far as we know, there is no single target organ for the growth hormone and the question of the control of the anterior pituitary by the hormones secreted by the target organ therefore does not arise. But a discussion involving the anterior pituitary gland would be incomplete without reference to the growth hormone, and I feel I can take my place, without undue embarrassment, in consideration of the influence of this anterior pituitary hormone on carbohydrate metabolism. Even so I shall have to restrict my field to a discussion of two main points: the so-called diabetogenic action of growth hormone, and certain aspects of the influence of growth hormone on protein deposition—protein of course being an important source of carbohydrate during normal metabolism. Finally I hope I shall be allowed to introduce two minutes of speculation concerning the possible general significance of the growth hormone on metabolic processes in general.

In our investigations concerning the diabetogenic activity of anterior pituitary extract we have always preferred to use the intact dog or the intact cat as test animal, and we have no

Haddow: I wonder if Dr. Lacassagne has considered the possibility that these specimens might be contaminated with traces of, say, stilbœstrol itself?

Lacassagne: After we had made our first experiment with PHP synthesized in our laboratory, the chemists found it took too long to prepare it for the clinical and experimental work, and they tried to obtain a commercial product. For several months we have done experiments with a preparation from the United States, and it was totally inactive. I have asked the chemists how it is possible that two products of the same chemical formula have such different activity. They could not reply It is possible that either in one or the other it is an impurity. I don't think that it is stilbœstrol. I am not an organic chemist, and I cannot discuss this question, but I have asked if it is possible It seems that it should not be. But what is the chemical difference between the two products, the products we received from America and the products we made in Paris, I cannot say.

Nowakowski I couldn't understand one point, why you have different effects on the gonads. In one slide you showed a gonad atrophy, but in another case, where I believe you gave the dosage by mouth, there was no atrophy of the gonads.

Lacassagne: I spoke about growing animals first; and then about adult mice. The growing animals are much more sensitive than adult mice. In a few weeks we can see the alterations that we have shown in the growing mice, but it is necessary to give the treatment for four months to obtain the same effects in the adult

Nowakowski In the treatment of exophthalmic goitre by p-hydroxypropiophenone, have the clinicians seen any atrophic changes in the gonads in these patients?

Lacassagne. I have no personal experience with the clinical work

Young: Was the food intake of your treated young growing rats smaller than that of the controls? Did they eat less?

Lacassagne There was no change. This substance is not toxic. We can maintain these animals given PHP more than one year.

Smelser I was discussing this work with Dr. Pierre Klotz in the Hôpital Bichat, Paris. He has had opportunities to treat six cases of malignant exophthalmos, with negative results. Unfortunately I did not know enough at the time to ask where he obtained the preparations he used. His comment in reference to one of the clinical papers, also from Paris, in which they had positive results in the treatment of exophthalmos with PHP was that in this case the treatment was instituted immediately after cessation of X-irradiation of the pituitary gland, and he felt that the physician had been a little too quick to conclude a negative effect of the pituitary irradiation and to institute a second therapy with this drug.

Bush. Has Professor Lacassagne tried the effect of gonadotrophin in the presence of these doses of PHP?

Lacassagne: No, we have not studied it. We have observed no effect on prolactin. For instance, mice receiving PHP 14 days before delivery could have young, and there was no inhibition of lactation.

example, precipitation under various conditions, treatment with acid and with enzymes, iodination and acetylation, all led to the disappearance of diabetogenic activity (in the intact cat) at the same rate as growth-promoting activity in the intact rat (Reid, unpublished observation). One interesting result of the acetylation of growth-hormone carried out by Dr. Reid is the finding that both growth-promoting and diabetogenic activities depend on the integrity of the ε-amino acid groups of the lysine residues in the peptide chain but not on the α amino groups of the alanine and phenylalanine residues at the ends of the chains.

These results suggest that the diabetogenic activity of the growth hormone is a property of the molecule itself, but since the diabetogenic activity of ACTH in the human being has been demonstrated by Conn and his colleagues and others in the U.S A , the possibility still exists that in the intact dog or cat small amounts of ACTH, which we suspect could lurk in the purest of growth hormone preparations, might exert a synergistic action with respect to the diabetogenic action of growth hormone. However, in a preliminary attempt to demonstrate the diabetogenic activity of ACTH in the intact cat we have completely failed. Even 50 mg./kg./day has proved to be ineffective in the cat, though 1–2 mg./kg./day of growth hormone is sufficient. Campbell, Davidson, Snair and Lei (1950) with the intact dog, and Houssay and Anderson (1949) with the partially depancreatized dog or cat have also failed to find a clear-cut diabetogenic effect of ACTH, although Ingle, Li and Evans (1946) were successful in the intact rat.

In spite of this lack of diabetogenic activity of ACTH alone in the dog and cat, Dr. Reid (1951) finds that it appears to exert some synergistic action with respect to the diabetogenic action of less purified preparations of growth hormone in the intact cat, although this effect is less well marked with more purified growth hormone. The significance of these observations is still obscure.

All the evidence available so far suggests that the diabetogenic action of growth hormone is a property of the molecule

difficulty in inducing diabetes in these species of animal without preliminary surgical or other interference with the pancreas. I emphasize this point particularly because we are beginning to realize that the secretion of insulin may not be the sole function of the islets of Langerhans of the pancreas, and in experiments in which partially depancreatized animals are used it may be unsafe to assume that the results are directly applicable to animals with the pancreas intact. In our experiments the minimal diabetogenic dose of purified growth hormone prepared by the method of Li or of Wilhelmi appears to be, for the cat, about 1 mg./kg. of body-weight/day (Cotes, Reid and Young, 1949) while that for the dog may be about twice this figure.

Since our publication numerous investigators have reported on the diabetogenic activity of purified growth hormone, some in the intact animal but mostly in animals from which the major part of the pancreas had been removed. It is interesting to note that the dose of purified growth hormone employed by Houssay and Anderson (1949) to induce diabetes in the non-diabetic partially depancreatized dog or cat was twenty-five to fifty times that we found to be needed in experiments in which the intact dog or cat was employed as test animal.

Naturally we have been anxious to know if diabetogenic activity is a property of the growth hormone itself (the assumption being made that the protein is indeed the growth hormone) or whether it is associated with a second type of molecule present as an impurity. Another but perhaps less probable alternative is that the two types of activity (growth-promotion and diabetogenesis) are to be ascribed to different structures in the same molecule. Dr. E. Reid at Cambridge has recently argued that if diabetogenic and growth-promoting activities do not depend on identical structures in the same molecule then any treatment of the protein which led to a change in its growth-promoting potency might result in a quantitatively greater or less change with respect to diabetogenic action. Numerous different types of procedure, for

metabolism, which is stimulated, or on carbohydrate and protein metabolism, which are depressed, or whether an effect is exerted on some or all of these simultaneously. We are however inclined to believe that the deposition of protein under the influence of growth hormone is of primary importance.

The possibility then arises that processes in which a positive nitrogen balance is needed may be particularly susceptible to stimulation by growth hormone. Certainly the production of milk by the lactating cow is increased by the administration of growth hormone (Cotes, Crichton, Folley and Young, 1949) although the results concerning a possible influence of treatment of a pregnant animal with growth hormone on the size of the offspring are inconclusive (Cotes, unpublished observations). Recent attempts by Miss Cotes to detect growth hormone in blood and urine from pregnant and lactating animals have been unsuccessful (Cotes and Young, 1951). Nevertheless we believe it is very probable that the physiological significance of the growth hormone is much greater than its name would imply

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itself. Since, however, in the young of all species we have examined, including the kitten and the puppy, growth hormone does not induce diabetes but growth (Young, 1941, 1949), it seems not unreasonable to suppose that the influence of growth hormone on carbohydrate metabolism is physiologically significant.

Dr. Greenbaum and I have investigated the site of deposition of the protein laid down in the normal rat under the influence of growth hormone, and have observed bizarre variations from one muscle to another which are hard to account for. Wondering whether these differences might be associated with differences in lability of the protein in different muscles, we have compared the behaviour of the same muscles during six days starvation, with that resulting from the action of growth hormone. The correlation is not good but the observations do suggest that with some exceptions, of which that of the masseter muscle is the most striking, the muscles which increase in size more under the influence of growth hormone are those which lose more substance under the influence of fasting (Greenbaum and Young, 1951). Recently Miss Bigland and Miss Jehring at University College, London, have investigated the work capacity of the quadriceps muscle of the growth-hormone treated rat and have found no functional differences despite an increase in weight up to 40 per cent (Bigland and Jehring, 1951). When expressed as g. tension/g. muscle the figures obtained from the treated muscles were significantly lower than those from their control counterparts. The probability that the hypertrophy of the muscle, amounting to 6–12 per cent increase in cross section area of the fibres, did not result from the predominant deposition of effectively contractile tissue is supported by the observation of Miss B. J. Hume at Cambridge, that the proportion of the total muscle protein present as myosin is not raised as the result of growth hormone hypertrophy.

... do not know yet whether the influence of growth ... ...ted on fat

Gurin's insulin might have contained some glycogenolytic factor. Whether that would explain the difference between our results I don't know.

HOET: In connection with the variability of the diabetogenic action of growth hormone in different species and in the same species at different ages, as shown by Professor Young, I should like to point out the greatly increased diabetogenic action of cortisone in the Addisonian patient, as seen in these two cases of Addison's disease, treated with 100 mg. cortisone (Table I) In cases without chronic

Table I

DIABETOGENIC ACTION OF CORTISONE IN ADDISON'S DISEASE

Blood Sugar Values after Administration of 50g Dextrose				
		Before Dextrose	*After 45 minutes*	*After 90 minutes*
M P. 54 years old, weight 45 kg B.P 90/60 Addison's for 3 years Treated with DCA	Before treatment	0 88	1 27	0 89
	8/2/51 24 hours after 100 mg cortisone	1 26	2 80	1 66
	9/2/51 (100 g dextrose)	1 66	2 04	2 13
	11/2/51	2 05		2 34 6 p m
S A 40 years old 52 kg Never treated	Before cortisone	0 89	1 77	1 67
	4 days 100 mg cortisone	1 31	2 66	2 32 (Urine 21 g %)
	2 days after discontinuing cortisone	1 14	2 00	1 90

DISCUSSION

LI: Two experiments which we hope to publish soon may substantiate Professor Young's hypothesis that growth hormone is a hormone with diabetogenic activity. Our *in vivo* and *in vitro* experiments on the glycogen formation and glucose uptake of the rat diaphragm suggest that part of the explanation of the diabetogenic activity of growth hormone is perhaps a direct action in antagonizing the function of the insulin molecule. The second experiment was done in collaboration with Dr. D. J. Ingle of Upjohn Laboratories. He wished to find out whether glycosuria can be produced in the normal rat by growth hormone. A number of years ago we were able to produce a glycosuria with a high carbohydrate diet and a high dose of ACTH. In this experiment, with normal male rats on a medium carbohydrate diet, the growth hormone alone had no effect, but in the presence of ACTH there was some sort of synergistic action in the production of glycosuria.

YOUNG: A synergism between growth hormone and ACTH may also arise in our experiments with cats. Certainly the rat is a curious animal in that in our experience it doesn't develop diabetes at all with the growth hormone, even with very large doses. It is interesting that in this species addition of growth hormone potentiates the diabetogenic action of ACTH.

GRAY: Do you think there is any possibility that growth hormone exerts an effect on the absorption of glucose in the renal tubules? I'm just wondering if the results of Dr. Li's experiments could possibly be due to a lowered renal threshold. Did you in fact measure the blood glucose?

LI: No, we just measured the glycosuria.

GRAY: There is a possibility it could be due to an alteration in the renal pressure.

LI: It's quite possible. The growth hormone is known to affect the kidney function in some smaller animals.

YOUNG: We find that in the dog a high protein diet is much more effective in stimulating the production of diabetes under the influence of growth hormone than is a high carbohydrate diet. Have you tried giving a high protein diet?

LI: No. When we varied the diets, we found that with ACTH a high fat diet was more effective in producing glycosuria in the rat. Of course, this may be a species difference.

REID: I wondered if you have any comments on the fact that in Cori's laboratory purified growth hormone does not seem to have an *in vitro* effect on the diaphragm.

LI: I don't think anybody has found any *in vitro* effects on the diaphragm with growth hormone, as far as I know.

FOLLEY: With regard to the question of growth hormone and fat metabolism, at a previous conference here, Dr. S. Gurin reported (see *Isotopes in Biochemistry*, p. 283) that the incorporation of labelled acetate into fatty acids by liver slices was stimulated by insulin *in vitro*, and that the insulin effect was antagonized by growth hormone, also

sugar for some days after pancreatectomy. de Bodo's experiments are all acute, and undoubtedly some insulin may be present in the tissues of these animals. I have a prejudice that the action of growth hormone in inducing the deposition of protein is dependent upon the availability of insulin in the tissue At least I'd like to see it proved that there is no insulin available at all.

LONG: I would agree with that. The reason I showed the slide was [illegible]

thing, as you did in your earlier work.

suprarenal insufficiency the diabetogenic action of cortisone can be seen after many days, but the tolerance curve falls one hour and a half after dextrose. Therefore I suppose that in chronic suprarenal insufficiency of long standing, the pancreas is in a functional and perhaps morphological condition which makes the organ sensitive to the diabetogenic action of cortisone This increased diabetogenic action of cortisone has been seen in four other cases of Addison's disease. It is a regular fact seen in fresh cases or in cases treated with DCA implantation

LONG: I will show two slides [not reproduced] in an attempt to complicate this story further. They illustrate the differences between administration of growth hormone to fasted diabetic animals and fasted normal animals, the so-called paradoxical effect. In rats made diabetic by alloxan, the administration of highly purified growth hormone produces an increase in the blood glucose level. I'll call your attention to the fact, however, that there also occurs a fall in the amino nitrogen in the blood in spite of this diabetogenic effect. However, if the same amount of hormone is given to normal fasting rats, instead of a rise in the blood glucose we now get a marked hypoglycæmia, but this is still accompanied by a fall in the amino nitrogen of the blood Indeed, if this preparation is administered to adrenalectomized animals such a severe hypoglycæmia appears that the animals will usually die of its effects within an hour or so Here we have a paradoxical effect of the growth hormone, depending apparently on the presence or absence of the pancreas. Whether this means that growth hormone actually stimulates the secretion of insulin or whether it merely means that the growth hormone withdraws from the metabolic mixture such a large proportion of protein that the blood glucose can no longer be maintained at the normal fasting level, we do not know

YOUNG: Have you any observations with fully depancreatized animals? de Bodo and his collaborators recently demonstrated that in fully depancreatized animals growth hormone will produce a fall in blood sugar level

LONG No, we have not carried out this experiment on depancreatized cats and dogs, which presumably are the proper species for this However, our animals were quite severely diabetic, with a fasting blood glucose of nearly 300. That does not mean that there may not still be a little insulin present, but our experience has been, at least with the rat, that regardless of the severity of the diabetes produced by alloxan, we always observe a rise in blood glucose. We have not seen these immediate hypoglycæmic effects such as de Bodo has recently described in the dog soon after pancreatectomy.

YOUNG. In diabetic rats we can't demonstrate an effect of growth hormone either in causing a rise or fall in blood sugar. There seem to be some strain differences in this respect. And we've had a cat that has been persistently diabetic now for over a year, and to which small doses of growth hormone have been given at intervals, without any obvious effect one way or the other. I think these species differences are very important. I wonder whether the administration of growth hormone to the fully depancreatized animal would still produce a fall of blood

certain species the reproductive cycle was profoundly influenced by events in the reproductive tract. Thus, in the rat and mouse sterile mating through mechanical stimulation of the cervix (Long and Evans, 1922) throws the basic cycle out of gear, prolongs the life of the corpus luteum and causes a period of pseudopregnancy which would not otherwise appear. In the rabbit, even more dramatically, ovulation is dependent on mating, which exerts its effect by some means not connected with the seminal fluid or with mechanical stimulation of the vagina or cervix. I do not need to enlarge upon the steps by which the reconciliation has been effected or by which the hypothalamus has become implicated in a complex chain of stimulation, composed of both nervous and humoral links. I should, however, point out that the reproductive processes provide very good examples of the reciprocal co-ordination between the anterior pituitary body and the organs affected directly or indirectly by its secretions.

I have already referred briefly to the effects of X-irradiation on the ovary. If a mouse is exposed to an adequate dose of X-rays, drastic changes follow very rapidly in the ovary. The whole of the existing oocytes degenerate and come to form crumpled ghosts which may persist for a long time. The follicles, bereft of oocytes, break down and fade away. Corpora lutea, if existing at the time of the irradiation, are not affected, but disappear later in the ordinary way. In addition to all this, the germinal epithelium loses its capacity to re-form germ cells, so that in a very short time the ovary becomes completely devoid of live oocytes. The germinal epithelium may, however, subsequently bud off short tubes, which probably represent anovular primordial follicles, but which undergo no further development (Brambell, Parkes and Fielding, 1927) When irradiation is carried out on the immature animal the onset of puberty is not affected, and the basic 5-day cycle in the reproductive tract is established as usual (Parkes, 1926). When the adult animal is irradiated the cycle continues unaffected by the ensuing chaotic state of the ovary or by its subsequent non-cyclic nature (Parkes,

PART IV

GONADOTROPHINS

OVARIAN PERIODICITY IN THE ABSENCE OF CYCLIC STRUCTURES

A. S PARKES

An ovary from an immature animal grafted into an adult animal starts immediately to function as a mature ovary. Conversely, an ovary from a mature animal implanted into an immature animal ceases to function (Foa, 1900). Further, if one ovary is removed, the other undergoes compensatory hypertrophy and does the work of two ovaries. The activity of the ovary is therefore dependent on some extra-ovarian stimulus which is inadequate in the immature animal, and adequate in the mature one for a given degree of ovarian activity. Again, if the follicles and thence the corpora lutea are abolished from the ovary by X-irradiation, the basic cycle in the reproductive tract, dependent on the periodic action of ovarian œstrogen, is unaffected. The basic endocrine cycle of the ovary, like its over-all activity, is thus dependent on extra-ovarian control.

These observations and deductions were illuminated brilliantly by the discovery of the gonadotrophic hormones of the anterior pituitary body, but they belong to ancient history, and I mention them only because they bear on the new material which I have to describe. The discovery of the hypophyseal control of the ovary, however, while it explained one group of facts, had itself to be reconciled with another group of equally well known facts which indicated that at least in

were remarkably consistent; in each of the 10 animals a normal vaginal cycle was re-established within 13-19 days of making the graft, as would have happened if a normal ovary had been transplanted. Only one of these animals has so far been killed; the graft consisted of nodules of secretory tissue devoid of oocytes or follicles. The uterus was fully functional, which indicates that the vaginal cycle observed was not of the type reported by Bourne and Zuckerman (1940) to arise from a continuous œstrogenic stimulus at threshold level. This result indicates that freezing by the method employed was compatible with the survival of a large proportion of the secretory tissue of the ovary and that the absence of cyclic structures did not affect the basic cyclic activity of the ovarian tissue, which, as in the case of the irradiated mice, is presumably dependent on cyclic activity of the anterior pituitary body. It remains to find out whether mating in such animals, which would necessarily be sterile, would throw the basic 5-day cycle out of gear and lead to a condition of pseudopregnancy.

In the second group the ovaries were frozen slowly to −79°C. and stored at that temperature in most cases for about 10 days, but in one instance for 83 days; they were then thawed out and implanted back into the donor animal. Vaginal smears were not made immediately, but in each animal the vagina became atrophic, as after ovariectomy, and remained so for 2–3 months after the grafting of the ovarian tissue. Signs of œstrogenic activity then slowly became apparent, and vaginal smears made when the vagina was fully active showed a state of persistent cornification The delay in the establishment of effective endocrine activity on the part of the graft makes it very likely that there was massive destruction of the ovarian endocrine tissue on freezing and thawing by the method employed, and that the few surviving cells took a long time to build up a functional graft. Such greater destruction, as compared with that found in the other series of animals, may have been due to the difference in the temperatures employed or to other known differences in technique.

1927). The effect of sterile mating was not investigated specifically in these experiments on irradiation, and incidental observations on this point were inconclusive.

The new work which I want to describe to you has arisen as a side shoot of the study being made by my colleagues and myself on the survival of living cells at low temperatures, a study which so far has included spermatozoa, red blood cells, and certain endocrine tissues Dr Audrey Smith and I have found that ovarian tissue of the rat, chopped into small pieces and soaked in 15 per cent glycerol saline, can be frozen slowly to −79°C. (solid CO_2 in alcohol) or to −192°C. (liquid air) for long periods without destruction of all its components. Thus, ovarian tissue, so treated, implanted subcutaneously into the ovariectomized donor "takes" as a graft and sooner or later gives rise to typical œstrogenic effects. Such grafts, however, according to our present experience, are completely devoid of oocytes, and thence of follicles and corpora lutea. Oocytes present at the time of freezing are apparently destroyed, together with the germinal epithelium or at least its power of neoformation of oocytes.* The graft arising from frozen material is therefore very similar to the ovary sterilized by exposure to X-rays. Histologically, we have not observed the crumpled remnants of oocytes characteristic of X-irradiated ovaries, but the short tubules seen in irradiated material are prominent also in the frozen grafts. Functionally, the over-all picture also seems to be similar, but there are inherent differences in the techniques involved which are associated with suggestive differences in results.

There are two groups of rats, results on which I want to describe and contrast.

In the first group, the ovaries were frozen slowly to −192°C., kept at that temperature for 1 hour or 9 days, and then thawed out and implanted back into the donor animal Vaginal smears were made from the day of ovariectomy. The results

*These observations, made on long-term grafts, have been fully substantiated by later work. It appears, however, that in their early stages grafts regenerated from frozen tissue may reform a small number of oocytes and follicles.

whether the cycles he observed were œstrous cycles or vaginal cycles. Will these rats mate and show mating reflexes?

PARKES. Very recent experiments show that they will.

WILLIAMS It would be interesting to know whether the ones with constant cornification are continuously in œstrus or merely show continuous vaginal cornification. If there are, as Dr. Parkes said, possibly two types of cell in the implants, then one might produce œstrogen and the other progesterone

PARKES. I'm afraid the rats are dead now, the experiment was imperfectly designed from this point of view. What we were interested in was finding conditions of freezing compatible with permanent preservation of ovarian tissue, and other things emerged as sidelines If we get any more animals of that kind we shall certainly mate them, as we shall mate those that are now cycling.

HOUSSAY With regard to the production of vaginal cycles in spayed rats with daily injection of a constant quantity of œstrogens, these vaginal cycles are also obtained more intensely without the adrenals The idea that the adrenal was important was explained by Zuckerman, who has repeated this experiment in monkeys In some cases del Castillo and his fellow workers have obtained uterine cycles in castrated human beings by injecting constant doses of œstrogens.

PARKES. I think that's just one more difference between mice and men, that you don't get threshold uterine cycles in mice.

TUCHMANN-DUPLESSIS I wanted to ask you if you had studied the pituitary of these spayed animals with the grafts. You said there would be castration changes in the pituitary Are there castration

you explain this œstral cycle in which you see only cornification?

PARKES After the first session of this symposium on Monday, I realized we had committed a great error in not getting the pituitaries from those continuously cornified rats; the only excuse is that we were so excited at having got some sort of graft from tissue which had been left at -79°C. for weeks that we didn't think of it. But we shall do so in future

developed graft

LACASSAGNE When you published your experiments on radiation of the ovaries of mice, I was surprised at the difference in radiosensitivity of the ovary of the rabbit. You can totally sterilize the rabbit. I have obtained in mice exactly the same results as in the rabbit, by doing unilateral ovariectomy and irradiating the remaining ovary with a very narrow beam of X-rays, with high doses I obtained a true sterilization—total atrophy of the ovary, a total anœstrus.

Whatever the explanation of this anomaly, however, the fact that the delay was associated with the production, ultimately, of persistent cornification is highly suggestive. Lack of cyclic activity may be the ultimate fate of all such grafts, though there is no support for this idea in the records of the irradiation work, or it may have been due in the animals in question to unknown causes. Most likely, however, the delay in the graft becoming active, increased in one rat by the long interval between ovariectomy and grafting, resulted in castration changes taking place in the anterior pituitary gland. From the point of view of the present symposium the interesting point is that, on this explanation, the ovary devoid of cyclic structures was not able to restore the "castration" type of pituitary to cyclic activity. Be this as it may, further study of the ovary devoid of cyclic structures, in the light of modern knowledge of the pituitary-gonadal relationship, is likely to lead to instructive results.

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DISCUSSION

EVERETT: I would like to recall del Castillo's observation that after

threshold stimulus, then the adrenal will apparently cause some kind of vaginal cycle. However, the threshold stimulus for the uterus is many times higher than that for the vagina, and the uteri of the rats with an active graft were perfectly all right, so we're not dealing here with an adrenal cycle superimposed on a constant threshold stimulus from the ovarian graft.

WILLIAMS: I think that threshold cycles, vaginal cycles, are not œstrous cycles· there is no mating I should like to ask Dr. Parkes

INTER-RELATIONS OF GONADOTROPHIC AND GONADAL HORMONES IN THE REGULATION OF TESTICULAR FUNCTIONS

WARREN O. NELSON

THE important rôle played by the anterior hypophysis in testicular function has been amply confirmed by numerous studies since the pioneer investigations of Smith (1930). It has been shown that two gonadotrophic substances seem to be involved in the normal maintenance of the two testicular functions, production of germ cells and secretion of hormone. Although earlier studies, such as those of Smith, Engle and Tyndale (1934) and Greep, Fevold and Hisaw (1936) seemed to indicate clearly that spermatogenesis and androgen production are stimulated selectively and respectively by FSH and ICSH (LH), further investigations have provided reasons for doubting the existence of such clearly separable relationships. This is particularly true in the case of spermatogenesis.

Probably the first evidence that provided reason for examining these relationships more closely was the demonstration that urinary extracts containing androgenic substances (Walsh, Cuyler and McCullagh, 1934) and crystalline androgenic hormones (Nelson, 1937) would maintain spermatogenesis in hypophysectomized rats. The same results have been obtained in other species and it has been shown, furthermore, that while relatively small amounts of androgen will inhibit spermatogenesis, large amounts will maintain spermatogenesis, despite the fact that in either case the secretion of hypophyseal gonadotrophins is inhibited (see Ludwig, 1950, for discussion of this phenomenon). These studies showed that in some species, at least, androgen has a direct stimulating effect upon spermatogenesis and led to the conclusion that androgen plays a definite rôle in the process of

PARKES: You must have cooked them pretty well because in the ordinary way endocrine tissue is very resistant to X-rays, and I think other things would go first. How long do they live after that kind of dose?

LACASSAGNE: 583 days after 6000r in one case (Lacassagne, A., 1946 *Proc. R. Soc. Med.*, 39, 605). I can never obtain these results in trying to obtain bilateral sterilization by X-rays because the animals get lesions of the intestine and die.

spermatogenesis. In the absence of sufficient amounts of gonadotrophin the testes remain in the infantile state (Heller and Nelson, 1948*a*). When chorionic gonadotrophin is given in adequate amounts the Leydig cells are stimulated and androgen is secreted. The tubular response to such treatment in these cases is interesting. In those individuals whose testes before treatment, as revealed by biopsy, contained tubules without lumina and with germ cells in the spermatogonial stage, the response of the tubules was confined to a differentiation and enlargement of Sertoli cells with little or no change in the germ cells. On the other hand we have observed recently that in two cases where the original biopsy revealed considerable numbers of primary spermatocytes as well as spermatogonia, treatment with chorionic gonadotrophin (CG) induced the formation of spermatozoa. This observation suggests that CG, the active factor of which is akin to ICSH, has the capacity to carry spermatogenesis to completion from the primary spermatocyte stage. In the patients just mentioned it is believed, but not yet completely proven, that the deficiency of gonadotrophin was confined to the ICSH factor and that some FSH was present. In further support of this idea is our observation that the combined use of FSH and ICSH in all these hypogonadal men was followed by complete spermatogenesis

Some reports in the literature, for example the recent one of Barter, Sniffen, Simmons and Albright (1951), have indicated that the use of CG only is sufficient to induce spermatogenesis in hypogonadal men. These observations may be explained by our finding that CG will provide the only treatment necessary for spermatogenesis in hypogonadal men whose testicular tubules contain primary spermatocytes and who presumably are producing small amounts of gonadotrophic hormone, particularly FSH.

We have mentioned that Sertoli cells undergo differentiation under the influence of androgen secreted by the Leydig cells of hypogonadal men who receive CG. This apparent relationship between androgen and Sertoli cell differentiation is seen

sperm production. Indeed, the idea was advanced (Simpson, Li and Evans, 1944) that ICSH might be the sole gonadotrophin necessary for spermatogenesis. It should be noted, however, that more recently Evans and Simpson (1950) have found that spermatogenesis is stimulated in hypophysectomized rats by a pituitary preparation said to contain FSH only.

It would appear reasonable to suspect that both FSH and ICSH, the latter through the medium of androgenic hormone, may play a rôle in normal spermatogenesis, and at the present time this concept seems to most satisfactorily embrace and harmonize the accumulated evidence. As yet it is not possible to relate the individual hormones to precise rôles in the process and such is not likely to be possible until purification of the two gonadotrophic hormones has progressed further than at the present time. In an attempt to explain the respective rôles of FSH and ICSH in spermatogenesis Gaarenstroom and de Jongh (1946) have presented a theory that, when reduced to its essential points, would suggest the following (1) FSH increases the number of spermatogonia which have the capacity to undergo mitosis to form other spermatogonia and so increase the number of cells available for further maturation. (2) ICSH, through the medium of androgen produced in the Leydig cells, prolongs the life of the germ cells, thus permitting them to continue maturation. It is suggested that the later stages of spermatogenesis are not under hormonal control.

The first part of this theory, relating to the rôle played by FSH, would appear to be more defensible than the second. However, until accurate counts are made of mitotic activity in the spermatogonia of hypophysectomized animals, with and without replacement therapy with purified gonado- [illegible] it too must be accepted with reservations The same

Certain observations on human testes may be helpful in elucidating the rôles played by the hypophyseal hormones in

high sperm-producing potentials. However, it should be noted that in the two experiments which have been cited both the androgen and the route of administration as well as the rate of testicular activity were different.

Further evidence in favour of the participation of ICSH, through the medium of androgen, in the spermatogenic process has been provided by McCullagh (1948) who studied two eunuchoidal brothers whose sperm production was very low. Biopsy revealed poor spermatogenesis and apparent complete absence of Leydig cells. We have been privileged to study this material and more recently to encounter three additional cases of this testicular condition. In one of these the administration of CG caused a marked improvement in the production of sperm as well as the formation of Leydig cells and the secretion of androgen. In these cases it would appear that the hypophysis was secreting FSH but little or no ICSH, and that as a result of the deficiency of androgen spermatogenesis was unable to proceed normally.

In summarizing the inter-relations of gonadotrophic and gonadal hormones in man on the basis of available evidence it appears that FSH is important to the spermatogenic process, at least in providing a stimulus for continued production of spermatogonia and for the formation of primary spermatocytes Its relationship to further stages of sperm maturation is uncertain. Through the medium of androgen, ICSH seems to influence differentiation of Sertoli cells and may play an important rôle in the physiological activity of these cells. When both FSH and ICSH are present spermatogenesis can proceed to completion. Excessive amounts of androgen, and the same is true of œstrogen, interfere with spermatogenesis by inhibiting the secretion of hypophyseal gonadotrophin.

The mechanism controlling the secretion of gonadotrophin by the hypophysis is of great theoretical and practical interest. The idea advanced by Moore (1939) that the level of androgen circulating in the body exerts a controlling influence on the hypophysis has been the basis for many studies and their interpretation. Low levels of androgen were supposed to

in other conditions. We have had occasion to secure testicular biopsies in 6 boys, ranging from 3 to 6 years old, who showed sexual pseudoprecocity due to hyperplasia of the adrenal cortex. In 5 instances the Sertoli cells showed definite evidence of differentiation accompanied by tubular enlargement although in other respects the testes remained infantile.

That the response of the testis to gonadotrophins and to androgen may not be precisely the same in all species needs emphasis. It was disappointing to learn that the human testis does not show direct stimulation of spermatogenesis when large amounts of androgen are administered. In an attempt to duplicate the results obtained in laboratory animals we have treated men with 25 to 100 mg. of testosterone propionate daily for as long as four months. Biopsies obtained at the termination of treatment showed the complete cessation of spermatogenesis and the occurrence of severe peritubular fibrosis. However, it was gratifying to find that additional biopsies obtained 6 to 30 months later revealed a remarkable degree of spermatogenic recovery and the disappearance of fibrosis. Indeed, in almost every instance spermatogenesis was better than it had been prior to the initiation of treatment (Heller *et al.*, 1950) We have applied this finding, as have Heckel, Rosso and Kestel (1951), in certain cases of infertility. Sperm counts show a progressive drop in spermatogenesis during treatment, with a remarkable rebound, in most cases, to levels higher than those observed prior to treatment. It would appear that in man the parenteral administration of adequate amounts of androgen inhibits the secretion of gonadotrophin, with the result that sperm production suffers and that recovery occurs only when the administration of androgen is terminated. It is worth noting at this juncture that the administration of methyltestosterone linguets (20 to 40 mg. daily) for 30 to 45 days in 5 men whose sperm counts ranged between 150 and 270 million per ml. did not materially change their sperm counts. These studies require extension, but it appears that the administration of androgen has less effect on the pituitary-gonadal axis in men whose testes have

severe peritubular fibrosis of the human testis. This hypothesis, comparable to that offered in the case of the thyroid by Rawson, Sterne and Aub (1942), suggests that the level of

process of stimulating the gonads much of the gonadotrophins are destroyed or inactivated so as not to be detectable in the urine (Jungck, Heller and Nelson, 1947; Heller and Nelson, 1948b)

This concept does not deny the pituitary inhibiting action of the gonadal hormones since that phenomenon can be amply demonstrated in man and laboratory animals. It does, however, suggest that the amounts of gonadal hormone, particularly of androgen, which are required to effect inhibition of the hypophysis in man are beyond physiological limits. For example, it has been possible by implantation of pellets of testosterone to correct or alleviate conditions due to androgen deficiency in castrates, cases of the Klinefelter syndrome, and cases of the male climacteric, without lowering the elevated urinary levels of gonadotrophin that are present in these patients. While such evidence is by no means conclusive, it suggests strongly the existence of additional factors in the control of the secretion and excretion of gonadotrophins. It is possible that gonadotrophin inactivation in the

better explanation is provided for the existence of the increased levels of gonadotrophin than is offered by the inhibin hypothesis or the hypothesis which would hold that androgen alone controls the level of gonadotrophin secretion. In many cases of the Klinefelter syndrome, probably all cases of germinal cell aplasia, and the vast majority of cases of *peritubular fibrosis, there is no evidence of androgen deficiency.* In the remaining conditions, castration, cryptorchidism, and the male climacteric, as well as in some instances of the

encourage and high levels to inhibit secretion of gonadotrophin. However, this theory of reciprocal relationships between gonadotrophin and androgen has met with various objections. These stemmed first from the observation that the pituitary appeared to secrete more than normal amounts of gonadotrophin in cases where the production of androgen seemed reasonably normal, but where the spermatogenic process had been seriously damaged (X-irradiation, cryptorchidism, vitamin E deficiency). The idea was advanced that the testicular tubules produce a substance which is responsible for the maintenance of normal levels of gonadotrophin secretion and that in those conditions which interfere with spermatogenesis this substance is not produced. This hypothetical substance, originally believed to be water-soluble, has been given various names among which "inhibin" is the most familiar. McCullagh *et al.* (1950) have recently reviewed the evidence in favour of inhibin and should be consulted for further details.

It has been difficult for the proponents of the inhibin theory to determine the site of production, germinal or Sertoli cells, of the substance, although the basic observations would have pointed strongly toward the germinal cells. However, recent observations in two types of human testicular defects have led some people to modify the original concept. On the basis of observations on the levels of gonadotrophin, arbitrarily designated as FSH, in the urine of men with puberal seminiferous tubule failure (Klinefelter syndrome) and germinal cell aplasia, the idea was advanced that the Sertoli cells are responsible for the production of a pituitary inhibiting substance. This substance has been designated variously as "x-substance," pregnenolone and œstrogen (see Howard, Sniffen, Simmons and Albright, 1950; and Nelson and Heller, 1951, for further consideration of this subject).

An alternative hypothesis has been advanced to account for the occurrence of increased quantities of gonadotrophin in such conditions as X-irradiation, cryptorchidism, vitamin E deficiency, Klinefelter syndrome, germinal cell aplasia, and

may act to maintain the constant rate of secretion of gonadotrophin, but probably play a minor rôle except in cases where they are present in unusual amounts. In the event of loss of the testes or of failure of either or both of its components, tubules and Leydig cells, "utilization" of the gonadotrophic hormones is reduced and the urinary levels of these hormones are correspondingly increased.

Of related interest to the concepts that have been discussed is the question of the site of œstrogen production in the testis. Œstrogen has been found in the urine and testes of various species, including man, and in lesser quantities in castrates than in intact subjects. It has been generally assumed, primarily on the basis of the studies of Huggins and Moulder (1945) on tumours of the dog testis, that the Sertoli cells are the site of production of œstrogen (Teilum, 1950; Howard, Sniffen, Simmons and Albright, 1950) However, recent studies have suggested that, in the human species at least, the Leydig cells are the more likely sites of production than the Sertoli cells, and that the Sertoli cells are, as has been believed for many years, primarily, if not completely, sustentacular elements. Thus we have found that the gonads of five intersexes, ostensibly female, were testes with infantile characteristics except for the presence of large numbers of Leydig cells. The tubules were small and either lacked germ cells completely or contained very few. The Sertoli cells were undifferentiated in several of these cases and were not regarded as likely sources of the œstrogen which these gonads were producing (Nelson, 1951) Additional evidence has also been presented (Maddock and Nelson, 1951) in a group of adult men who were treated with CG. In each of these cases the urinary levels of œstrogen were markedly increased during the period of treatment. This period of increased excretion of œstrogen was accompanied by definite stimulation of Leydig cells without noticeable change in Sertoli cells. These findings suggest caution in regarding the Sertoli cells as the site of production of testicular œstrogen and as elements which are concerned with the control of gonadotrophin

Klinefelter syndrome, androgen deficiency does exist to a greater or lesser degree; but as noted earlier, the amount of androgen which is required to suppress the elevated levels of gonadotrophin in these patients is far in excess of that which will correct the manifestations of androgen deficiency.

The concept which maintains that the Sertoli cells produce some substance having a pituitary inhibiting effect cannot explain the occurrence of high levels of gonadotrophin in men with cryptorchidism, the male climacteric, and the germinal cell aplasia, since in these conditions Sertoli cells are present and appear to be normal by all tests that have been applied. Furthermore, no evidence has been offered that the hypothetical substance they are supposed to produce is decreased in these conditions or in cases of the Klinefelter syndrome or of peritubular fibrosis.

Inviting as the concept of gonadotrophin utilization may be, it can scarcely be regarded as more than a tentative hypothesis. Experiments must be done in which "utilization" of gonadotrophin can be measured directly and the urinary levels of gonadotrophin in the various types of conditions cited must be assayed for the two gonadotrophic factors. The latter procedure, hampered by the lack of satisfactory procedures for analysis of FSH and ICSH in urine, should show variations in the levels of the two hormones in the different types of testicular failure if the hypothesis is correct. Thus the urine of castrates should contain increased amounts of both FSH and ICSH while the urine of men with germinal cell aplasia should show an increase of FSH only.

Until adequate evidence is provided either in support of or against the concept of "utilization," the best explanation for the control of gonadotrophin levels in the male is provided by combining the better features of the different hypotheses. One might suppose that under ordinary circumstances the pituitary secretes gonadotrophins at a fairly constant rate. Most of these hormones are "utilized" by the gonads in the production of germ cells and gonadal hormones, i.e., androgen and œstrogen. The latter substances, particularly œstrogen,

may act to maintain the constant rate of secretion of gonadotrophin, but probably play a minor rôle except in cases where they are present in unusual amounts. In the event of loss of the testes or of failure of either or both of its components, tubules and Leydig cells, "utilization" of the gonadotrophic hormones is reduced and the urinary levels of these hormones are correspondingly increased.

Of related interest to the concepts that have been discussed is the question of the site of œstrogen production in the testis. Œstrogen has been found in the urine and testes of various species, including man, and in lesser quantities in castrates than in intact subjects. It has been generally assumed, primarily on the basis of the studies of Huggins and Moulder (1945) on tumours of the dog testis, that the Sertoli cells are the site of production of œstrogen (Teilum, 1950; Howard, Sniffen, Simmons and Albright, 1950). However, recent studies have suggested that, in the human species at least, the Leydig cells are the more likely sites of production than the Sertoli cells, and that the Sertoli cells are, as has been believed for many years, primarily, if not completely, sustentacular elements. Thus we have found that the gonads of five intersexes, ostensibly female, were testes with infantile characteristics except for the presence of large numbers of Leydig cells. The tubules were small and either lacked germ cells completely or contained very few. The Sertoli cells were undifferentiated in several of these cases and were not regarded as likely sources of the œstrogen which these gonads were producing (Nelson, 1951) Additional evidence has also been presented (Maddock and Nelson, 1951) in a group of adult men who were treated with CG. In each of these cases the urinary levels of œstrogen were markedly increased during the period of treatment. This period of increased excretion of œstrogen was accompanied by definite stimulation of Leydig cells without noticeable change in Sertoli cells. These findings suggest caution in regarding the Sertoli cells as the site of production of testicular œstrogen and as elements which are concerned with the control of gonadotrophin

secretion. The studies do not, of course, deny the possibilit[y] that œstrogen produced in the testis has an effect upon th[e] secretion of gonadotrophins by the pituitary gland.

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DISCUSSION

'ed with highly purified
is, and myself (*Endo-*
low dosage there is no
definite stimulation of
tubules and spermatogenesis Even with high dosage the interstitial cells still atrophy, but the tubules are stimulated I was wondering whether the rat is different from the human, and that that difference

togenesis, but whether or not it alone can carry spermatogenesis on to complete maturation I am not as yet convinced. We have not ourselves

further in commenting on that point.

Sertoli cell stimulation, as far as we can determine, appears to be effected to some extent through the Leydig cells. Many types of

Nelson has expressed.

Nelson: In his original publication del Castillo said that these men had normal gonadotrophins, but I talked to him in Atlantic City recently and he's not so certain at the present time In our experience,

Leydig cells are not functioning normally.

NELSON: So far as we're able to tell, the first focus of failure in the Klinefelter syndrome centres in the tubules, but the Leydig cells ultimately fail too. Whether this failure occurs early in puberty or later will determine the condition of the patient. If the failure occurs early, coincident with the tubular failure, then those individuals will be eunuchoidal. If it occurs late or occurs post-pubertally, then those individuals will be essentially normal physically; they'll have normal genitalia, normal hair development, normal stature. But the Leydig cells usually do fail even in these cases at a relatively early age, and in that event treatment with androgen proves beneficial in relieving the symptoms of androgen deficiency It is correct, I believe, to classify cases of the Klinefelter syndrome as eunuchoidal, moderately eunuchoidal and non-eunuchoidal in accordance with the physical condition of the individuals.

GONADOTROPHIN EXCRETION IN MAN

P. J. BANKS, H. M. LLOYD and E. F. SCOWEN

Previous comparisons of the gonadotrophic activity of urinary extracts prepared by precipitation in alcohol and by ultrafiltration through collodion membranes have indicated that the two methods yield roughly similar amounts of hormone (Gorbman, 1945; Jungck, Maddock and Heller, 1947). However, during investigations of gonadotrophin excretion by human subjects we have found that differences in activity of the two types of extract frequently occur. The variations in activity with the two methods of extraction are observable in response to the exhibition of gonadal hormones but are much more striking in response to stilbœstrol.

It would appear that the findings must represent either a variation in anterior lobe response or in mode of excretion. The failure to demonstrate such a type of change in men with diminished or absent anterior lobe function would indicate that the variations in gonadotrophin excretion represent variations in anterior lobe response. We wish to draw attention firstly to circumstances in which there is divergence in the activity of the two types of extract, and secondly to those in which the two yields are similar.

The method of precipitation in alcohol described by Klinefelter, Albright and Griswold (1943) has been adopted, and dialysis and reprecipitation in alcohol have been carried out in all cases. The technique of ultrafiltration is that of Gorbman (1945). The activity of the extracts has been determined by biological assay, the increase in weight of the uterus of the immature female mouse being the criterion of the test.

Post-menopausal women excrete large amounts of gonadotrophin which can be extracted both by precipitation in alcohol and by ultrafiltration. Case 4 indicates the effects of

stilbœstrol on this excretion (Fig. 1). This was a woman of 52 years, suffering from osteoporosis, the menopause having occurred spontaneously at 36 years. Initial excretion levels were high by both methods of extraction. During the admini-

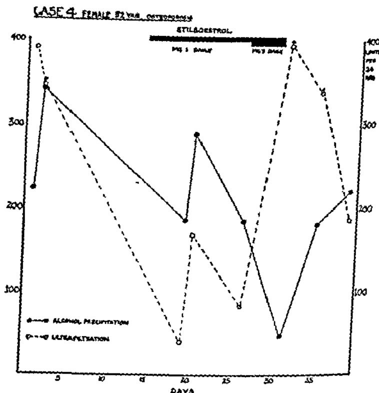


FIG. 1 Excretion by post-menopausal woman, showing response to stilbœstrol, with divergence in activity of alcohol precipitate and ultrafiltration extract.

stration of stilbœstrol mg. 1 daily, there was a fall in activity of the ultrafiltration extract to 40 units on the sixth day of treatment, while the activity of the alcohol precipitate remained high. On the next day, there occurred a return of activity in the ultrafiltration extract to 170 units/24 hours. The dose of 1 mg. daily was continued for 12 days, and then

for five days, 5 mg. daily was given. On the day following withdrawal, excretion was 433 units/24 hours by the ultrafiltration method, and 49 units/24 hours by alcohol precipitation. During the subsequent ten days there was a return to levels of about 200 units in both extracts. Here then is an example of two different responses in excretion of gonadotrophin—firstly a depression of activity of the ultrafiltration extract, accompanied by high activity of the alcohol precipitate, and secondly a highly active ultrafiltration extract accompanied by a weakly active alcohol precipitate on the day following cessation of stilbœstrol.

In a post-menopausal woman the yields from the two extraction methods showed no wide differences. This patient, 67 years of age, was suffering from thyrotrophic exophthalmos which had developed eight years previously, nine years following subtotal thyroidectomy for toxic goitre. Also there was a mild diabetes mellitus, for which no insulin was required. Five initial gonadotrophin assays showed that excretion was varying from less than 30 units to 128 units/24 hours, the higher activity being present in the alcohol precipitate only. The activity of the ultrafiltration extracts did not rise above 60 units. Stilbœstrol 5 mg. daily was given for five days, and after the third dose the ultrafiltration extract showed increased activity (107 units) which was still present two days later. On these two occasions the alcohol precipitate showed activity of 50 and 30 units. A fall in excretion then occurred, and it remained low during the next twelve days following cessation of stilbœstrol. A very marked increase in activity of both extracts was then found, and this was maintained four days later. After the first dose of a second course of stilbœstrol, a fall to less than 50 units occurred, the activity of the two extracts being closely similar. On the next day, however, ultrafiltration (80 units) gave a greater yield than alcohol precipitation (36 units). Excretion remained at a low level on days 38, 42 and 44. Thus here the gross change in activity after withdrawal of stilbœstrol was shown in both extracts, and apart from the

slightly increased ultrafiltration yield during stilbœstrol administration the two methods gave similar results throughout.

A third post-menopausal woman (aged 68 years) who was suffering from osteoporosis, was studied in relation to the effect of œstradiol. The administration of œstradiol benzoate

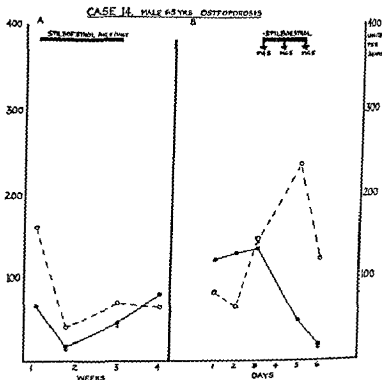


FIG. 2 Excretion by 65-year-old man, showing response to stilbœstrol.

3 mg. every fourth day resulted in no significant change in excretion after 3½ weeks. The two extracts were of similar activity on both occasions. No reaction was therefore detected to this gonadal hormone.

Divergence of results has been encountered in male subjects, and is particularly marked during administration of synthetic

œstrogen. Case 14 (Fig. 2A) was a man of 65 years suffering from osteoporosis. Initially gonadotrophin excretion was high, but there was no other evidence of gonadal deficiency. Stilbœstrol 5 mg. daily was taken for two weeks. On the first day, the activity of the ultrafiltration extract was 160 units/24 hours, which was significantly greater than that of the alcohol precipitate (66 units). The remaining results showed a fall in activity of both extracts with possibly greater depression of the alcohol precipitates. One week after the cessation of stilbœstrol, the activity of the alcohol precipitate was increased.

When the same subject was studied again nine months later, stilbœstrol mg 5 daily was given for three days (Fig. 2B). After the third dose, a rise to 239 units/24 hours occurred in the ultrafiltration extract, whereas the activity of the alcohol precipitate was depressed. On the next day ultrafiltration gave a yield of 122 units/24 hours and alcohol precipitation less than 18 units. Divergence was therefore demonstrated in a man with increased gonadotrophin excretion, and the increased activity under the influence of stilbœstrol was present in the ultrafiltration extract only.

More marked differences in yield by the two methods were demonstrated in a series of five men who were receiving stilbœstrol (Fig. 3). Four were suffering from prostatic carcinoma. Case 9, 79 years of age, had taken stilbœstrol for six years, and at the time of assay the daily dose was 20 mg. Ultrafiltration gave a yield of 270 units/24 hours and alcohol precipitation 38 units Case 10, 61 years of age, had received stilbœstrol 15 mg. daily for three years. Assay was performed on two occasions, and on each the activity of the ultrafiltration extract was much in excess of the alcohol precipitate. Case 11, 68 years old, had for three years taken 1–3 mg daily and the dose at the time of assay was 1 mg. The yields were 80 units by ultrafiltration and 24 units by alcohol precipitation. Case 12, 59 years old, after seven days on stilbœstrol 20 mg., gave widely divergent results, the alcohol precipitate showed no detectable activity, whereas ultrafiltration gave a value of

464 units/24 hours. In this last subject excretion values were also obtained before treatment, and were found to be low by both methods. Stilbœstrol was administered for six days to a man of 63 years with benign prostatic hypertrophy. The first dose was 10 mg. and the remaining five 20 mg. On the

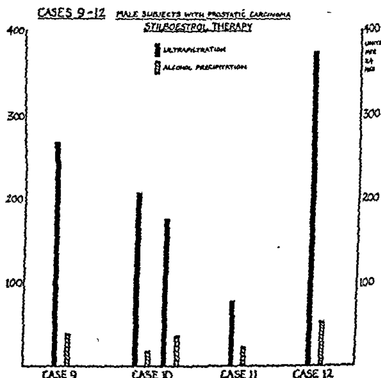


FIG. 8. Excretion by four men receiving stilbœstrol, showing highly active ultrafiltration extracts and inactive alcohol precipitates.

third day excretion was 17 units (alcohol) and 389 units (ultrafiltration) and on the fourth day 34 units (alcohol) and 163 units (ultrafiltration). Three weeks after the withdrawal of stilbœstrol, the activity of the ultrafiltration extract had fallen to 60 units, and the last result, three days later, was 20 units. The alcohol precipitate was only slightly active on these two occasions (14 and 17 units).

This behaviour of five men in relation to stilbœstrol administration may be contrasted with that of the next three cases.

Administration of stilbœstrol to two men with hypogonadism, low gonadotrophin excretion and anterior lobe failure resulted in little change in the activity of the extracts. Thus Case 16, 46 years, received stilbœstrol 5 mg. daily for 11 days and no rise in the activity of the ultrafiltration extract occurred. Case 15, 57 years, showed no detectable gonadotrophin excretion by either extraction method (less than 12 units/24 hours) until the fifth of a five day-course of stilbœstrol 5 mg. daily. Ultrafiltration then gave a yield of 80 units/24 hours and alcohol precipitation less than 25 units. The stilbœstrol was discontinued, and three days later, the two extracts showed no activity. Case 6 was a woman of 72 years, suffering from hypertension, in whom the menopause had occurred at 51 years of age. Gonadotrophin excretion was low initially and remained low throughout the administration of stilbœstrol 0·5 mg. on alternate days, followed by two doses of hexœstrol 2 mg.

Finally, conditions where there is depression of gonadotrophin yield by either extraction method in post-menopausal women can be illustrated. Cases 1 and 2 (Fig. 4) were given 50 mg. testosterone propionate daily, and depression of activity of both extracts occurred. It must be noted that the ultrafiltration extract of Case 2 during the second week of treatment showed no activity, in contrast with the high activity of the alcohol precipitate. The combined effect of testosterone propionate and œstradiol benzoate was also investigated in Case 2. During the last week of testosterone administration, three doses of œstradiol mg. 2 were given. Both hormones were discontinued, and two weeks later the assay figures were 210 units for alcohol precipitation and less than 18 units for ultrafiltration. The next assay after an interval of four days showed that the activity of the ultrafiltration extract had risen to 159 units/24 hours. Thus although depression occurred in both extracts during testosterone therapy and during

combined testosterone and œstradiol administration, the release effect occurred at different times, being first shown in the alcohol precipitate.

Dietary restriction was found to be effective in reducing activity of both extracts in the cases of two post-menopausal

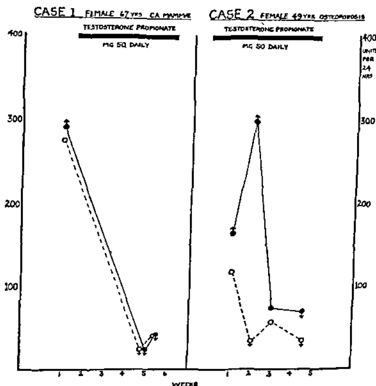


FIG. 4 Excretion by two post-menopausal women, showing response to testosterone with depression of activity of both extracts.

women. Case 7 was an obese woman of 60 years, and while she was receiving a normal diet yields were high by both extraction methods. For two weeks the diet was reduced to two apples and five pints of fluid, and then increased to 750 calories. Four days later, no activity was detectable in either extract. Case 8, 54 years, suffered from obesity, and activity

of both extracts was initially high. Diet was then reduced to 750 calories, and six weeks later the activity of the alcohol precipitate was considerably diminished (125 units) and that of the ultrafiltration extract completely depressed. The reaction to stilbœstrol 0·5 mg. daily is interesting in that at the end of the third week, ultrafiltration gave a yield of 125 units, while the activity of the alcohol precipitate showed no significant change.

Summary

Comparisons have been made of gonadotrophic activity of urinary extracts prepared by ultrafiltration and precipitation in alcohol

Conditions in which the two types of extract show (i) similar and (ii) different gonadotrophic activity have been investigated.

Administration of stilbœstrol to male and female subjects was associated with gross fluctuation in excretion, and marked divergence in activity of the two types of extract.

Stilbœstrol administration to two men with anterior lobe failure resulted in little or no alteration in gonadotrophin excretion.

By either extraction method, it was found that the gonadotrophin excretion of post-menopausal women was depressed by administration of testosterone propionate and by restriction of diet.

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DISCUSSION

CROOKE. I should have thought that this might have been explained purely on technical grounds. We have used both these methods and have generally got much better yields from alcohol precipitation than from ultrafiltration. I wonder whether you're getting a complete

separation with your method. Another possible cause of discrepancy may be in the number of mice used.

LLOYD: We have used immature female mice weighing initially between 7 and 11 grams, ten animals per dose level, and the unknown preparation is compared with a preparation of pregnant mares' serum as a standard. The answers are expressed in terms of units of pregnant mares' serum per 24 hours.

CROOKE: We now rely entirely on the kaolin precipitation method which was developed in Professor Gaddum's laboratory, and we think [illegible]

alcohol precipitates on a large number of normal patients who had not received any hormone preparations, and although we have found differences on occasions, we have not found such marked differences as in the examples I have shown you of subjects receiving œstrogen therapy, particularly male cases receiving stilbœstrol.

QUERIDO: May I ask a technical point? We have the impression that you might encounter differences when you extract the residue which you have obtained. Our impression is that if you leave it at room temperature for an hour or if you extract it in the ice box it makes a great difference in the results, and I wondered what method you used?

LLOYD: We have adopted the same technique throughout our use of ultrafiltration. The membrane is dissolved in equal parts of absolute alcohol and absolute ether, and the final precipitate is then allowed to dry; it is extracted before assay (an hour before assay as a rule) with tap water at room temperature.

QUERIDO. Have you ever tried dividing the residue and using different temperatures of extraction?

LLOYD: No, we haven't done that.

SCOWEN: As far as the technique is concerned, it's identical throughout, and since the divergence comes in two different reactions, I can't see how you could possibly explain this on the basis of precipitation alone.

[illegible]

[illegible]

down with the ultraprecipitate, and would be taken up; whereas in the alcohol precipitate of course it would remain in solution in the alcohol. That could possibly account for the greater results, because you were

that isn't the high dosage that some of the other patients were given.

Swyer: The biggest discrepancies were in cases receiving 20 mg., and quite clearly there may be differences even in the same patient from

get different slopes, for one thing, and so the actual comparison is apt to be rather fallacious

Lloyd: We have naturally made comparisons of these substances before we used this assay routinely, and although these results will take a lot of going over, we were satisfied initially as regards the assay, and it was a help to have some substance of a standard nature.

PART V

THYROTROPHIC HORMONE

FACTORS WHICH INFLUENCE THE PHYSIOLOGICAL REACTIONS OF THE THYROID-STIMULATING HORMONE OF THE PITUITARY

RULON W. RAWSON

MANY physiological and pathological states of thyroid function have been attributed to either an increased or a decreased secretion of the thyroid-stimulating hormone (TSH). From these theories there has evolved a concept known as the pituitary-thyroid axis, which suggests a finely balanced relationship between the thyroid and the pituitary, in which a decreased level of circulating thyroid hormone is balanced by a compensatory increased secretion of TSH, and conversely an increased level of circulating thyroid hormone results in a decreased secretion of TSH. Observations which indicate that certain ovarian or adrenocortical hormones, when in excess, inhibit the thyroid have resulted in a broadening of the axis, to include the gonads and the adrenals. The thyroid-inhibiting properties of gonadal and adrenal hormones have been interpreted by some investigators as phenomena resulting from a decreased pituitary secretion of TSH.

Although we know of no direct evidence that hormones of the gonads or of the adrenal inhibit the production of TSH, we believe that there is ample evidence in support of the hypothesis that an excess of thyroid hormone will decrease and even abolish the production of TSH by the pituitary. Likewise, the evidence for a decreased thyroid function

resulting in an increased production of TSH, though indirect, is strongly suggestive. We should like to present an additional theory that certain physiological and pathological changes in the thyroid are related to factors which influence the thyroid's response to TSH.

This theory was first suggested to us by a study of thyrotrophic substances in the urine of humans who presented various states of thyroid function (Rawson and Starr, 1938). In that investigation it was observed with the microhistometric method of assay that euthyroid males excrete small but measurable amounts of thyrotrophin. No thyrotrophic activity was found in the urine of patients having untreated hyperthyroidism, whereas relatively large amounts of thyrotrophic substances were demonstrated in the urines of three recently totally thyroidectomized patients Furthermore, we have observed that certain people with low basal metabolic rates and presumably colloid goitres excrete increased amounts of thyrotrophic substance. These observations are not inconsistent with the thesis that an increased level of thyroid hormone inhibits the secretion of TSH by the pituitary, whereas an absence or decreased level of thyroid hormone results in an increased secretion of TSH. However, the theses suggested to us were that the thyroid in responding to TSH inactivated the hormone and that the amount of TSH inactivated depended on the state of the thyroid or other body mechanisms which influence the thyroid's response to the stimulating influence of the pituitary.

The theory that a functioning thyroid inactivates its trophic hormone was first suggested by the observations of Loeser (1934), who administered intravenously to intact and to thyroidectomized rabbits large amounts of pituitary extracts rich in thyrotrophic hormone. He then assayed the blood of his animals for thyrotrophic activity at various intervals. He was unable to demonstrate any thyrotrophic hormone in the blood of his intact rabbits sixty minutes after administering the extract. However, he was able to demonstrate this hormone in the blood of his totally thyroidectomized rabbits as

PART V

THYROTROPHIC HORMONE

FACTORS WHICH INFLUENCE THE PHYSIOLOGICAL REACTIONS OF THE THYROID-STIMULATING HORMONE OF THE PITUITARY

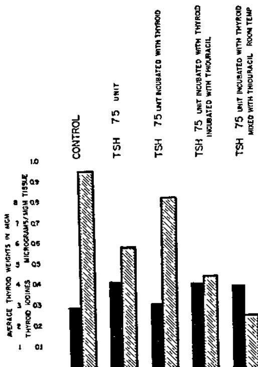
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dehydrogenating enzyme system and is thus inactivated; (2) that the thymus and lymph nodes are also target organs of the thyroid-stimulating hormone.

Studies by Dr. Sonenberg now in progress in our laboratory give some evidence for the latter theory. Dr. Sonenberg, as he told you this morning, is labelling with isotopic techniques various pituitary hormone preparations and then following



It is to be noted that TSH exposed to thyroid tissue was

temperature.

long as seven hours after administering the pituitary extract. Seidlin (1940) did a similar study in intact and thyroidectomized guinea pigs to which he administered a preparation of thyrotrophic hormone. He was able to demonstrate thyroid-stimulating substances in the urine of thyroidectomized guinea pigs but none in the urine of intact animals.

In our studies (Rawson, Sterne and Aub, 1942) we utilized tissue culture techniques in evaluating the effects of thyroid and other tissues on the thyroid-stimulating properties of pituitary extracts. Explants from young adult rabbits of thyroid and control tissues, i.e., adrenal, kidney, liver, ovary, pancreas, parathyroid, spleen, stomach mucosa, testis, thymus, and lymph nodes, were bathed for 72 hours at 38°C. in Tyrode's solution containing a pituitary extract rich in thyroid-stimulating hormone, in amounts equivalent to 3·5, 7·5 and 15 Junkmann-Schoeller units per explant of tissue. Following such exposure the media were removed and assayed for thyroid-stimulating properties by the microhistometric method of Rawson and Starr (1938).

It was observed with these methods that explants of thyroid weighing between 60 and 100 mg. were capable of inactivating as much as 12 units of TSH and that similar explants of thymus and of lymph nodes were capable of inactivating nearly half as much. None of the other tissues studied were capable of inactivating the exposed TSH. Furthermore, by studying the effects of exposed and unexposed pituitary extracts on the testes of cockerels we have demonstrated that the thyroid does not inactivate gonadotrophic hormone. We have also observed that thyrotrophic hormone inactivated by exposure to explants of thyroid tissue can be reactivated by exposure to minute amounts of strong reducing agents which are also goitrogenic, i.e., thiouracil, 5-amino-2-mercaptothiadiazole, or 3-phenyl amino methyl-2-mercaptothiazoline (see Fig. 1).

From these observations we have developed two theories: (1) that the thyroid-stimulating hormone in exerting its action on its target gland contributes to the metabolism of the thyroid cell through some unidentified oxidative or

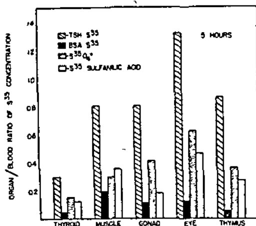


FIG 2a

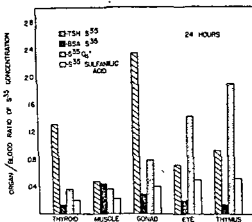


FIG 2b

FIGS. 2a and 2b The localization of ^{35}S-labelled TSH, beef serum albumin (BSA), sulphate and sulphanilic acid in chick tissues The concentrations of the various labelled agents

the fate of such labelled pituitary preparations in various experimental animals. He is labelling his thyrotrophic hormone preparation with ^{35}S labelled diazobenzenesulphonic acid. Such labelled preparations of TSH are injected intravenously into chicks. The chicks are then sacrificed at varying intervals and the radioactivity in various tissues is compared with that in whole blood. Similar observations are made in animals which have received beef serum albumin labelled in a similar fashion, with ^{35}S labelled sulphate or ^{35}S labelled sulphanilic acid. At the five-hour interval Sonenberg has found the organ/blood ratio of ^{35}S labelled TSH in the thyroid to be twice that of the control ^{35}S preparation. At this interval he has found an even greater concentration of radioactive sulphur in muscle, gonads, orbital contents, and the thymus of animals which received the labelled hormone preparation (see Fig. 2*a*). At twenty-four hours the concentration of radioactive sulphur in the thyroids of the hormone-treated chicks has been found to have increased fourfold over the five-hour level, whereas the concentration in muscle had decreased to the level of the control ^{35}S preparation. At the same interval there was also a significant decrease in radioactive sulphur in the orbital controls of the hormone treated chicks. The level in the thymus remained about the same as at the five-hour interval. The radioactivity in the gonads of the hormone-injected chicks had increased even more than it had in the thyroid at the 24-hour interval and was higher at all time intervals than in any other tissues (see Fig. 2*b*). This latter observation is not surprising when one considers that our preparation of TSH is very rich in gonadotrophic activity. As pituitary preparations are far from being pure it is impossible to make any statement as to other specific end organs of the thyroid-stimulating hormone. However, the fact that the thymus, which inactivates TSH, picked up large amounts of this preparation rich in thyrotrophic hormone whilst it did not pick up other labelled pituitary hormone preparations, suggests strongly that the thymus is a target of thyrotrophic hormone.

This theory is supported by our observation (Money and Rawson, 1950) that the thyroids of rats subjected to prolonged (18 to 27 months) treatment with thiouracil no longer show the extreme diffuse hyperplasia so characteristic of the thyroids of rats which have been under shorter periods of treatment with thiouracil, but show the picture of a colloid goitre surrounded by a rim of hyperplastic tissue. We believe that the large colloid-filled follicles, lined by flat and inactive-appearing epithelium, represent exhausted thyroid tissue which is no longer capable of responding to the secreted TSH.

An increased sensitivity to thyrotrophic stimulation has been described in laboratory animals under various experimental conditions by Starr *et al.* (1936), Stephens and Allen (1941), Soffer *et al.* (1949), and by Money *et al.* (1951). Starr, Patten and Brunner reported in 1935 that the thyroids of guinea pigs whose ovaries at time of sacrifice showed large corpora lutea were more sensitive to the administration of a preparation of TSH than were the thyroids of similar animals whose ovaries presented active follicles. Stephens reported in 1941 (Stephens and Allen, 1941) that the thyroids of guinea pigs on a weight-losing diet were considerably more sensitive to TSH than were the thyroids of similar animals on a weight-gaining diet. He was unable to alter this situation by administering any of the known vitamin preparations. Soffer and his associates and Money in our laboratory have observed that the administration of epinephrine to intact rats results in a decreased capacity of the thyroid to collect radioactive iodine However, in the adrenalectomized rats treated with epinephrine they have observed a greater than normal capacity of the thyroid to collect radioactive iodine. In view of Soffer's demonstration that the administration of epinephrine to dogs results in an increased secretion of TSH, the above observations on rats may be interpreted as representing an increased sensitivity of the thyroid to thyrotrophic stimulation in the absence of certain adrenal steroids.

In another study Money (Money *et al.*, 1951) has investigated the effects of several steroids of gonadal and adrenal

Further studies on the inactivation of TSH have been carried out with human thyroid tissue, normal and pathological (Rawson, Graham and Riddell, 1943). Explants of human thyroid tissue weighing between 150 and 200 mg. were bathed in nutrient media (Tyrode's solution) containing 7½ Junkmann-Schoeller units of TSH. Following a 72-hour exposure, samples of the bathing media were removed and assayed for thyrotrophic activity in cockerels by the microhistometric method. The thyroids of patients with Graves' disease were removed after a maximum response to treatment with iodides had been obtained. The pre-treatment basal metabolic rates of these patients ranged between plus 25 and plus 65 per cent. Non-toxic nodular goitre tissue was obtained from routine operations for such goitres. The basal metabolic rates of these patients were all below minus 10 per cent. Samples of normal thyroid tissue were obtained as biopsy specimens at operations for parathyroid tumours. In these studies it was observed that 150–200 mg. explants of normal human thyroid tissue were capable of inactivating between 3 and 4 units of TSH. Similar explants of thyroid tissue taken from patients operated upon for Graves' disease inactivated between 6 and 7·5 units of the hormone. Since the histological picture of most of the thyroids taken from this group of patients showed involution, and in some instances hyperinvolution, we do not believe that this greater degree of inactivation can be attributed to cell mass.

We have suggested that the increased capacity of thyroid tissue in Graves' disease to inactivate TSH might explain our inability to recover active thyroid-stimulating hormone from the urine of patients suffering with untreated Graves' disease. We also suggest that the increased reactivity between TSH and the thyroid tissue of Graves' disease presents a clue to the pathogenesis of this disease.

The fact that the colloid goitres which we studied were incapable of inactivating TSH suggests that such goitres occur because of their inability to respond to the stimulation of thyrotrophic hormone.

This theory is supported by our observation (Money and Rawson, 1950) that the thyroids of rats subjected to prolonged (18 to 27 months) treatment with thiouracil no longer show the extreme diffuse hyperplasia so characteristic of the thyroids of rats which have been under shorter periods of treatment with thiouracil, but show the picture of a colloid goitre surrounded by a rim of hyperplastic tissue. We believe that the large colloid-filled follicles, lined by flat and inactive-appearing epithelium, represent exhausted thyroid tissue which is no longer capable of responding to the secreted TSH.

An increased sensitivity to thyrotrophic stimulation has been described in laboratory animals under various experimental conditions by Starr *et al.* (1936); Stephens and Allen (1941), Soffer *et al.* (1949); and by Money *et al.* (1951). Starr, Patten and Brunner reported in 1935 that the thyroids of guinea pigs whose ovaries at time of sacrifice showed large corpora lutea were more sensitive to the administration of a preparation of TSH than were the thyroids of similar animals whose ovaries presented active follicles. Stephens reported in 1941 (Stephens and Allen, 1941) that the thyroids of guinea pigs on a weight-losing diet were considerably more sensitive to TSH than were the thyroids of similar animals on a weight-gaining diet. He was unable to alter this situation by administering any of the known vitamin preparations. Soffer and his associates and Money in our laboratory have observed that the administration of epinephrine to intact rats results in a decreased capacity of the thyroid to collect radioactive iodine. However, in the adrenalectomized rats treated with epinephrine they have observed a greater than normal capacity of the thyroid to collect radioactive iodine. In view of Soffer's demonstration that the administration of epinephrine to dogs results in an increased secretion of TSH, the above observations on rats may be interpreted as representing an increased sensitivity of the thyroid to thyrotrophic stimulation in the absence of certain adrenal steroids.

In another study Money (Money *et al.*, 1951) has investigated the effects of several steroids of gonadal and adrenal

origin on the weight and capacity to concentrate radioactive iodine of the thyroid in rats maintained on an iodine deficient diet. The steroids were administered for ten days. The animals were sacrificed on the eleventh day, twenty-four hours after the intra-peritoneal injection of a tracer dose of radioactive iodine. None of the gonadal steroids studied altered the thyroid weights. However, the thyroids of rats treated with testosterone propionate (total dose 50 mg.) and œstrone (total dose 500 micrograms) were observed to collect significantly more ^{131}I than did their controls.

The thyroids of rats treated with ACTH or cortisone had lower absolute thyroid weights as well as lower thyroid/body weight ratios. Deoxycorticosterone in daily doses of 15 mg. and cortisone in daily doses of 2·5, 5·0 and 10 mg. caused a significant decrease in uptake of ^{131}I by the thyroid.

In another experiment we have studied the effect of ACTH and of cortisone on the thyroid's response to TSH in hypophysectomized male rats. Treatment with ACTH or cortisone was started three days following hypophysectomy. Cortisone was administered in a daily dose of 5 mg. for 4 days and then of 2·5 mg. for three days. ACTH was administered in doses of 3 mg (Armour's standard) three times a day for four days, and then 1·5 mg. three times daily for three days. TSH was administered in a dose of 1 mg. (6–8 chick units) once daily for the last four days of the experiment. One microcurie of ^{131}I was administered to each animal twenty-four hours after the last injection of TSH and the animals were sacrificed twenty-four hours later. The responses to TSH were evaluated by determining thyroid weights, mean acinar cell heights and the pickup of ^{131}I.

Whereas the effects of TSH on the thyroid weights and mean cell heights were not greatly influenced by the administration of cortisone or ACTH, both hormones caused a significant decrease in the effect of TSH on the collection of ^{131}I by the thyroids of these rats. From these observations and similar ones which have been reported (Woodbury *et al.*, 1951) it is suggested that at least part of the inhibition of

thyroid function produced by adrenal steroids is a direct one on the collection of iodine by the thyroid. At the present time we are unable to support or refute the theory that adrenocortical hormones inhibit the secretion of TSH by the pituitary.

Since the thyroid hormone is capable of inhibiting thyroid function via the pituitary and by a direct action on the thyroid, it would not surprise us to find both direct and indirect effects of the adrenal steroids on the thyroid.

We have observed that the administration of thyroxine in daily doses of 0·1 mg. to intact adult rats for three to four weeks results in a demonstrable loss of TSH from the pituitaries. We have also observed (Cortell and Rawson, 1944) that the administration of thyroxine to hypophysectomized rats results in a decreased response of the thyroid to physiological amounts of TSH. In one such experiment 20 micrograms of DL-thyroxine were administered subcutaneously for 10 days to hypophysectomized male rats. Thyrotrophic hormone was then administered in daily doses of 4 Junkmann-Schoeller units for the last four days of the experiment. The responses to TSH were estimated by determining the mean thyroid acinar cell heights and by measuring the thyroid's avidity for radioactive iodine. The mean thyroid acinar cell heights of the untreated controls averaged 2·6 micra, of rats treated with TSH 6 5 micra, of rats treated with thyroxine and TSH 4·8 micra. The percentage collection of the administered radioactive iodine by unoperated untreated controls averaged 6 9 per cent, of hypophysectomized untreated controls 0·4 per cent, of hypophysectomized rats treated with TSH 7 8 per cent, of similar rats treated with thyroxine and TSH 2·7 per cent (see Fig. 3). In this study we have evidence that thyroxine may inhibit the action of physiological amounts of TSH. In another experiment done on intact rats it has been observed that prolonged treatment of rats with doses of thyroxine, great enough to put the thyroid in a state which histologically is almost identical to that of hypophysectomized rats, increases the sensitivity of the thyroid to large doses of

thyroid-stimulating hormone. In this study thyroxine was administered to adult intact rats in a daily dose of 0·1 mg for 21 days. During the last four days of the experiment three groups of the thyroxine treated and of the untreated rats received TSH in daily doses of 0, 20 and 50 Junkmann-Schoeller units, respectively. The animals were sacrificed on

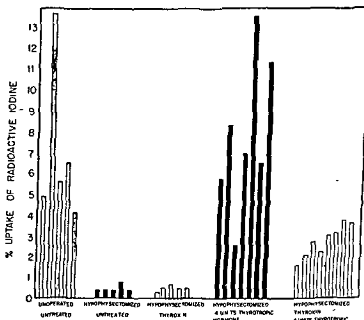


FIG. 3. The effect of thyroxine and TSH on the collection of ^{131}I by the thyroids of hypophysectomized rats.

the day following the last injection of TSH and the mean acinar cell height of each animal's thyroid was determined. Whereas the rats which had received no thyroxine made practically no response to TSH when administered in a daily dose of 20 units, they did show a very slight increase in mean cell height following the administration of 50 units daily. On the other hand, the mean acinar cell heights of animals which had been treated with thyroxine only averaged 3·2 micra, while similar animals treated with thyroxine and

TSH showed a very marked increase in mean cell height. Rats treated with 20 units daily had average mean cell heights of 7·73 micra and those treated with 50 units daily had an average mean cell height of 7·97 micra.

We have made a similar observation in one human who presented himself to the clinic with thyrotoxicosis factitia. He concentrated no radioactive iodine in the thyroid but had a protein-bound iodine of 21 micrograms per cent. Following the administration of TSH (100 Junkmann-Schoeller units daily for 7 days) he concentrated 70 per cent of a tracer dose of ^{131}I in the thyroid

There are also certain pharmacological agents which appear either to alter the circulating thyrotrophic hormone or to affect the hormone's action on the thyroid.

The very striking goitrogenic properties of chemical agents such as thiouracil and its derivatives have been widely attributed to an increased secretion of thyrotrophic hormone following inhibition of thyroid hormone production. There is no doubt that these agents interfere with the utilization of iodine in the synthesis of thyroid hormone and that the goitrogenic action of these agents is dependent upon an intact pituitary gland. It has also been demonstrated that those animals made goitrous as the result of treatment with these agents have pituitaries which are larger than normal and contain no demonstrable TSH, in contrast to the pituitaries of normal rats or of rats treated with thiouracil and thyroxine, in which approximately one chick unit of TSH can be demonstrated per pituitary.

However, we have not been fully satisfied with the above explanation of the goitrogenic action of these agents. For example, we have observed a fifty per cent increase in the mean acinar cell height in the thyroids of rats which have received thiouracil in the drinking water for only 24 hours. Furthermore, in these same animals we have failed to demonstrate any significant fall in the serum protein bound iodine levels at this 24-hour interval. With these observations it is difficult to explain the rapid increase in mean cell height as

the result of increased secretion of TSH secondary to a fall in circulating thyroid hormone.

In view of these observations we have proposed the theory that these agents act to augment the action of TSH. Support for this thesis was obtained (Albert *et al.*, 1947*a*) by administering active TSH, and TSH which had been inactivated by elemental iodine for three days, to cockerels receiving a standard diet and to others receiving a diet which contained 0·2 per cent thiouracil. It was observed that the thyroid weights of chicks receiving thiouracil and active TSH averaged 9·1 mg., in contrast to weights of 6·5 mg. in cockerels treated with active TSH and no thiouracil. In the chicks which received no thiouracil and inactivated TSH the thyroid weights averaged 3·7 mg. whereas the inactivated TSH when administered to cockerels receiving thiouracil in the diet produced thyroid weights which averaged 5·8 mg

Gassner (Gassner *et al.*, 1950) has extended these studies and has observed that propyl thiouracil has an even greater augmentary effect on administered TSH than has thiouracil. He has also demonstrated that 5-iodo-thiouracil and 5-iodopropylthiouracil are lacking in this capacity to augment the action of TSH even though they do manifest thyroid inhibiting properties. Since we have been able to augment the action of TSH by simple incubation of the hormone with minute amounts of thiouracil (Albert *et al.*, 1947*b*), we strongly suspect that this *in vivo* enhancement of TSH by thiouracil and related agents is due to a direct effect on the circulating hormone rather than to any direct effect of these drugs on the thyroid cell.

The important rôle that iodine plays in the synthesis of thyroid hormone is well established. The mechanism by which it exerts its therapeutic effects when administered to patients suffering with Graves' disease remains a point of disagreement among clinicians and physiologists. We have demonstrated that the involuting effects of *iodides* on the hyperplastic thyroid of Graves' disease can be separated from the nutritional effects of iodine (Rawson *et al.*, 1945). This was done

by comparing biopsy specimens taken from the thyroids of four patients with Graves' disease before treatment, and after treatment with thiouracil had brought about a fall in the basal metabolic rate to a normal level, with the histological pattern of several specimens taken from the thyroid removed subtotally after treatment with thiouracil plus iodides.

In the pretreatment biopsy specimens we observed the classic thyroid hyperplasia of Graves' disease and by micro-histometric measurements observed mean cell heights which averaged 18·2 micra Biopsy specimens removed after treatment with thiouracil had lowered the basal metabolic rates to normal, showed similar hyperplasia and an average mean acinar cell height of 14·0 micra. These same thyroids, removed after continued treatment with thiouracil and added iodides, showed involution and a marked decrease in cellular hypertrophy. The mean acinar cell heights averaged 8·0 micra. Notwithstanding these involutionary changes which were induced by iodides there was little or no iodine retained in these thyroids, as evidenced by radioiodine excretion studies and by the very low thyroglobulin iodine values in the operatively removed thyroids (see Fig 4).

We have postulated that this involution of such hyperplastic and hyperfunctioning thyroids occurs as the result of an inhibition to the reactions which occur between thyroid cells and thyrotrophic hormone (Rawson and Money, 1949). Support for this hypothesis is obtained from two different studies. In one we compared the mean thyroid acinar cell heights of hypophysectomized rats treated for four days with TSH, eight Junkmann-Schoeller units daily, with those of similar rats treated with the same amount of TSH and also sodium iodide in daily doses of 500 to 1,000 micrograms. The mean acinar cell heights of thyroids taken from untreated control rats averaged 8·8 micra, of the thyroids taken from rats treated with TSH 10·1 micra, from rats treated with TSH plus 500 and 1,000 gamma daily of sodium iodide 5·4 and 5·2 micra respectively.

In another study we have investigated the effects of iodide on the *in vitro* inactivation of TSH by explants of thyroid tissue. Explants of rabbit thyroid tissue were bathed in 15 ml. of Tyrode's solution containing 7½ units of TSH and sodium iodide in a concentration of 100 micrograms per 100 ml. At the same time similar explants of thyroid tissue were bathed in media containing the same amount of TSH but no sodium iodide. The degree of inactivation of TSH was estimated by microhistometric methods applied to the thyroids of chicks which had been treated with the variously exposed media. Complete inactivation of the hormone resulted from exposure to slices of thyroid tissue, whilst in the presence of sodium iodide very little inactivation occurred. We have also observed in the urine of a few patients with Graves' disease, who excreted no active TSH before treatment, significant amounts of thyroid-stimulating hormone after short periods of treatment with iodide. On the basis of these observations we can postulate that iodides inhibit the oxidative enzyme through which TSH makes its contribution to the metabolism of thyroid tissue.

Finally we should like to suggest that certain carcinogens act by altering the thyroid's response to the growth promoting properties of TSH and thereby promote the development of cancers in thyroids which are subjected to prolonged stimulation by TSH.

We have observed that the administration of thiouracil to rats for six to eighteen months promotes the development of a variety of tumours (Money and Rawson, 1950). None of the tumours which we have produced by the administration of thiouracil without any other form of treatment have been observed to metastasize nor have they been transplantable. However, when similar rats were treated with thiouracil and subcutaneously administered dibenzanthracene, tumours developed which histologically were similar to those observed in rats which had received thiouracil only. However, these tumours were transplantable. The mechanism by which this carcinogen affected such a change in response to the growth



FIG 4 The effect of thiouracil and of thiouracil and iodine on the thyroids of a patient with Graves's disease. On the left is a biopsy specimen of an untreated patient, BMR plus 40 In the centre is a biopsy specimen taken from the same patient after treatment with thiouracil had induced a fall in BMR to plus 8 On the right is a representative histological section from the thyroid of the same patient after continued treatment with thiouracil and added iodine. It is to be noted that iodine induced an involution of the thyroid, notwithstanding the fact that thiouracil was being administered in a dose great enough to prevent the incorporation of iodine into thyroglobulin

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DISCUSSION

TUCHMANN-DUPLESSIS. I would like to ask you what you think about the relationship between the adrenal and the thyroid. You said that the thyroid would be somewhat impaired after adrenalectomy. I thought it also a year ago, because I had a series of 20 rats which showed a decreased activity of the thyroid after adrenalectomy. But since then I have adrenalectomized more than 60 rats and I have never been able to see any histological modification of the thyroid. On the other hand, I have tried to study the influence of adrenal hormones on the structure of the thyroid, and I found many variations. With DCA I think that one can say that there is never any modification from the histological point of view. With cortisone, if you give very high doses there may be some inhibition, but I don't know whether this modification has a physiological or only a pharmacological meaning.

RAWSON: This is a problem that's been close to my heart for a long time We have found that the thyroids of intact animals of the Sprague-Dawley strain treated with adrenaline have a decreased pick-up of radioactive iodine, even in the immature female. (We had observed previously that the thyroid of the Sherman strain immature female rat did have an increased reaction to thyrotrophic hormone, as measured by cell height, after the administration of one dose of adrenaline). In 1939 we studied the effect of one stimulating dose of testosterone on the thyroids of immature female rats, and we observed that such a provocative dose would increase the cell height of the immature rat's thyroid from around 3 5μ to about 5 5μ in 96 hours. However, in the castrated adrenalectomized or adrenalectomized immature rats given ... observed a greater stimulus

animals I believe suggests that in the absence of the adrenal the thyroid is probably more sensitive to stress Then we have observed also that deoxycorticosterone in doses of 15 mg. a day does inhibit the collection of iodine by the thyroid, and cortisone in doses of 2 5 to 10 mg. a day also inhibits the collection of iodine by the thyroid

VAZQUEZ-LOPEZ: How did you apply the carcinogen in these experiments?

PENETRATION OF LABELLED THYROXINE INTO THE HYPOPHYSIS: RECENT FINDINGS

R. COURRIER

ADMINISTRATION to an animal of thyroid hormone in excess puts the thyroid gland itself into a resting phase (Herring, 1917; Cameron *et al.*, 1921, 1922; Courrier, 1922, 1924). Further, after treatment with thyroxine, radioactive iodine enters only very slightly into the thyroid gland (Joliot, Courrier, Horeau and Süe, 1945).

The mechanism by which thyroxine inactivates the thyroid gland is a matter of dispute. It is certain that thyroxine acts on the anterior lobe of the pituitary, and in order to discover whether this action is direct or indirect we have made use of labelled thyroxine, introducing ^{131}I into diiodothyronine. We began our researches in 1944 (Joliot, Courrier, Horeau and Süe), and made some new experimental findings in collaboration with Horeau, Morel and Marois (1949, 1951).

The thyroxine which we used had a stronger specific radioactivity; at the time of the experiment 1/10,000 μg. gave 70 counts per minute by the Geiger counter. The purity of this labelled thyroxine has been confirmed by radiochromatography, which shows radioactivity concentrated in the one spot characteristic of thyroxine.

We injected the labelled thyroxine into the marginal ear veins of rabbits. Other rabbits were given labelled solutions of KI* or of Na*Cl. The animals were sacrificed 2 hours later and the findings were as follows:—

(1) Male rabbits weighing 2 kg. were given 25 μg. labelled thyroxine per kg.; after two hours the ratio of radioactivity of 1 mg whole hypophysis/1 mg blood was 1·6–2·3. The ratio being more than 1 suggests that there has been concentration in the hypophysis.

(2) In male rabbits weighing 2 kg. and given KI*, with the same quantity of iodine of the same radioactivity as in *experiment* 1, the ratio of radioactivity of 1 mg. whole hypophysis/1 mg. blood was 0·55.

The two iodine containing substances, therefore, behave differently in regard to the hypophysis.

(3) In male rabbits weighing 2 kg. given Na*Cl, the ratio of radioactivity of 1 mg. whole hypophysis/1 mg. blood was 0·53.

Sodium is an extra-cellular element and it is seen that the ratios for sodium and ionic iodine are the same. It seems, therefore, that ionic iodine does not enter the parenchyma of the hypophysis, although the iodine of thyroxine becomes concentrated in the gland.

We have confirmed that it is thyroxine itself which enters the hypophysis and not a breakdown product containing the ^{131}I; we have been able to recover the labelled thyroxine from the glands by more than one procedure.

We have attempted to determine the difference between the intra- and extra-cellular fractions of the labelled thyroxine in the whole hypophysis by reasoning along these lines:—

With radioactive sodium, the ratio of radioactivity of 1 mg. hypophysis/1 mg. blood was 0·5. Now, this radioactivity represents that of the extra-cellular component of the fluids in the gland, and this component is found to have apparently the same activity as the blood. The ratio found, therefore, indicates that in 1 mg. of hypophysis there is *approximately* ½ mg. of extra-cellular fluid and ½ mg. of cellular components. Let us now consider the following experiment:—

A rabbit is given 25 μg. per kg. of labelled thyroxine. The activity of the thyroxine at time T is 26 counts per minute for 1/1,000 μg. After 2 hours, examinations and estimations are *made*:—

The hypophysis weighs 20 mg. Its total radioactivity is 116 counts per minute adjusted to time T. One mg. of circulating blood gives 4·5 counts per minute at time T. The hypophysis which weighs 20 mg. contains 10 mg. of extra-cellular fluid,

which corresponds to a radioactivity of $10 \times 4 \cdot 5 = 45$ counts per minute at time T. The activity of the cellular component at time T is, therefore, $116 - 45 = 71$ counts per minute. This means that $\frac{71}{26} = 2 \cdot 7$ thousandths of a microgram of thyroxine have entered the gland in 2 hours.

Other similar experiments have given the following results (Table I).

Table I

Labelled thyroxine injected /kg rabbit (in µg)	*Radioactivity 1 mg hypophysis/ 1 mg blood*	*Intracellular thyroxine in hypophysis in 1/1,000 µg after 2 hours*
5	2·6	0·6
25	2	3·1
250	2·3	29
250	2·3	12·3
500	0·7	10
1200	0·8	20

It is seen that in rabbits more than 250 µg. thyroxine per kg. saturates the pituitary parenchyma in 2 hours. The ratio of radioactivity of 1 mg. hypophysis/1 mg. blood becomes less than 1, because when the hypophysis is saturated, only the proportion in the blood is increased by larger dosage.

We repeated these experiments in rabbits, measuring separately the radioactivity of the anterior and posterior lobes. Male rabbits of about 2 kg were given 50 µg. of labelled thyroxine per kg. into the marginal vein of the ear Two hours afterwards, we obtained the following results, on average: for the anterior hypophysis, radioactivity of 1 mg. hypophysis/1 mg blood=0 58; for the posterior hypophysis radioactivity of 1 mg hypophysis/1 mg. blood=4·38.

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is, surprisingly, in the posterior and not the anterior that thyroxine appears to be concentrated, although the one acts without doubt on the morphology and physi- of the anterior lobe. This finding may be of importance nsidering the physiology of the whole hypophysis.

The research work which we have just considered has been carried out on the rabbit. Gross and Leblond (1947) did not find a selective concentration of thyroxine in the hypophysis of the rat and we have, therefore, done similar experiments in a variety of species.

Table II

Species	*Thyroxine injected µg./kg.*	*Anterior lobe/blood*	*Posterior lobe/blood*	*Whole hypophysis/blood*
Cat . .	50	0·42	0·42	0·43
Guinea pig	150	0·48	0·47	0·48
Rat . .	150	0·53	0·85	0·57
Cock . .	150	0·74	0·93	0·80
Monkey .	50	0·41	1·67	0·97
Rabbit .	50	0·58	4·38	1·38

With Na*Cl we obtained the following results:—

In the rabbit—anterior lobe/blood = 0·65; posterior lobe/blood = 0·69. In the rat—anterior lobe/blood = 0·57; posterior lobe/blood = 0·67. It is clear that the results vary with the species of animal.

We should like to report finally that we have not found concentration of thyroxine in the testis, submaxillary gland, myocardium, adrenal, nor in lymph glands. Animals injected with labelled thyroxine excrete the radioactive iodine in bile and urine.

In conclusion, of the organs studied the only one in which concentration of thyroxine has been found is in the hypophysis. The results vary with the species. In the animals in which we found this phenomenon, it was in the posterior lobe that most of the thyroxine was found to have concentrated during the two hours.

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DISCUSSION

HOUSSAY Have you determined the thyroxine in the hypothalamus or the tuber cinereum?

COURRIER: Yes, we have. We found no thyroxine in the hypothalamus.

SONENBERG: Was the administered thyroxine *lævo* or *dextrolævo*?

COURRIER. Racemic Diiodothyronine from Hoffman LaRoche.

SONENBERG: What chemical techniques did you use to identify the radioactivity as thyroxine?

COURRIER Isotope dilution, radiochromatography on paper, and butanol extraction.

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Table II

Species	*Thyroxine injected µg /kg*	*Anterior lobe/ blood*	*Posterior lobe/ blood*	*Whole hypophysis/ blood*
Cat	50	0 42	0 42	0 42
Guinea pig	150	0 48	0·47	0·48
Rat	150	0·53	0·85	0 57
Cock	150	0 74	0 93	0 80
Monkey	50	0 41	1 67	0·97
Rabbit	*50*	*0 56*	*4 38*	*1·38*

With Na*Cl we obtained the following results:—

In the rabbit—anterior lobe/blood = 0·65; posterior lobe/blood = 0·69. In the rat—anterior lobe/blood = 0·57; posterior lobe/blood = 0·67. It is clear that the results vary with the species of animal.

We should like to report finally that we have not found concentration of thyroxine in the testis, submaxillary gland, myocardium, adrenal, nor in lymph glands. Animals injected with labelled thyroxine excrete the radioactive iodine in bile and urine.

In conclusion, of the organs studied the only one in which concentration of thyroxine has been found is in the hypophysis. The results vary with the species. In the animals in which we found this phenomenon, it was in the posterior lobe that most of the thyroxine was found to have concentrated during the two hours.

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949). C.R.

what I am afraid is a very minor point—namely some evidence obtained a few years ago, and which we have been unable to extend, on the pathogenesis of that form of exophthalmos produced in the guinea-pig within a few days of starting daily pituitary injections in young animals, using acid or crude alkaline extracts of pig anterior pituitary. I want to specify this because changes causing this acute exophthalmos, which is the form that has been studied mainly, may be irrelevant to the exophthalmos occurring later after the start of injections in the guinea-pig.

May I first summarize briefly certain earlier observations (Pochin, 1944). Since the guinea-pig's eyes project laterally, a measurement of the intercorneal distance can be used to estimate the amount of exophthalmos, provided allowance is made for any change in total body weight from growth or wasting, which also influence this measurement. As body weight increases in the growing animal, the intercorneal distance increases correspondingly, and with loss of weight the intercorneal distance falls. The exophthalmos caused by pituitary injections, however, causes the intercorneal distance to increase out of proportion to the body weight, which may indeed fall. If therefore intercorneal distance is plotted against body weight, rather than each against time, the exophthalmos can be detected and estimated independently of concurrent changes in body weight. When determined in this way the exophthalmos could be shown to increase rapidly, reaching a maximum after a few days, then decrease slowly. It is perhaps of interest that the exophthalmos was equal in normal and in thyroidectomized guinea-pigs in these experiments, using this method to allow for the greater weight loss, which tended to mask the exophthalmos in the normal group after injection.

The amount of the exophthalmos could be related, in a roughly quantitative fashion, with the changes in orbital tissue bulk which caused it. This holds also for the enophthalmos associated with loss of body weight and confirms what would be anticipated, that the phenomenon may be more accurately

THE MECHANISM OF EXPERIMENTAL EXOPHTHALMOS CAUSED BY PITUITARY EXTRACTS

E. E. POCHIN

Exophthalmos provoked by anterior pituitary extracts has been shown to occur in a number of species, including the fish *Fundulus* that Albert and Money studied, and probably also in the duck and the pig. Most work upon it, however, has been done in the guinea-pig, where a massive exophthalmos can consistently be produced and of which Smelser (1939, 1943*a*, 1943*b*) has made a detailed analysis.

I think that those who have examined the exophthalmos in guinea-pigs were at first interested in the light that it might throw upon the exophthalmos that occurs in human Graves's disease, and therefore upon the cause of this condition. It was clearly important to see whether the exophthalmos and thyroid stimulation in the guinea-pig and in man might both be due to the anterior pituitary. At present there are many defects in the argument. Firstly, the evidence that anterior pituitary extracts cause exophthalmos in man is, I think, very tenuous, but this may be due simply to the low dosage that has been given in man compared with that needed, on a weight for weight basis, to provoke exophthalmos consistently in the guinea-pig Secondly, the local orbital changes responsible for the exophthalmos in human thyroid disease are not understood: excess orbital adipose tissue is found in simple exophthalmos and œdema and fibrosis, but no excessive adipose tissue in so-called progressive or malignant exophthalmos. The pathogenesis of these changes is still a matter for speculation. And thirdly, the way in which pituitary extracts cause orbital volume changes even in the guinea-pig is not fully explained. This is my reason for wishing to discuss

Histologically the shrinkage of both glands is associated with a reduction in size of the alveoli and enlargement of the central lumen (Fig. 2), strongly suggesting the output of secretion from a stimulated gland. The normal appearance (Figs. 3 and 4) is converted at 6 hours to one of shrunken

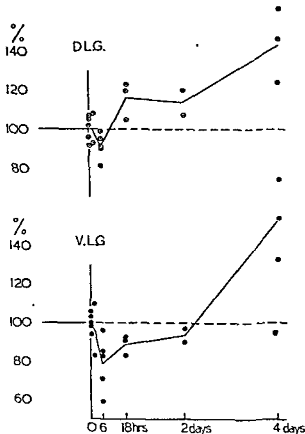


Fig. 1. Weight changes of [illegible] and
[illegible] of
[illegible] of

studied by examining the orbital tissues themselves. When this was done it was found that, at the height of the exophthalmos, the two lacrymal glands that occur in the guinea-pig were considerably increased in bulk, particularly the dorsal or Harderian gland, the capsule of which was sometimes greatly swollen to form a thick gelatinous mass. These two glands constitute most of the total retrobulbar tissues and it is difficult to establish whether other orbital tissues are enlarged, as the swollen gland capsule is intimately related to the eye muscles. The exophthalmos at this stage is due predominantly to lacrymal gland enlargement and Smelser (1941) has described the hyperplasia of the Harderian gland at this stage and its secretory overactivity, on which I wish to present further evidence.

These are the changes at the height of the exophthalmos several days after the first injection.

May I deal now with the changes observed immediately after the first injection. When the intercorneal distance is measured within a few hours of the injection the eyes are found to be enophthalmic, although only to a small degree, and this condition persists for about 6 hours after a large dose. The eyes then return to normal and exophthalmos develops, usually being detectable within 24 hours. During this initial enophthalmic phase it is often possible to see on the surface of the conjunctiva at the lid a collection of slightly refractile liquid which probably represents accumulated lacrymal secretion.

The changes in bulk of the lacrymal glands in these first hours run parallel to and presumably account for the changes in position of the eye (Fig. 1). Both lacrymal glands are found to be reduced in size soon after the injections, before later enlargement develops. The change is not large, but when measured in groups of injected animals and compared with control groups, it is significant and consistent, the reduction being greater and continuing longer in the ventral gland than in the dorsal or Harderian, in which the subsequent swelling is greater.



Fig 3. Ventral lacrymal glands before (C), and 7½ hours, 24 hours and 96 hours after start of daily anterior pituitary injections.

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alveoli with large lumens; later, pale staining cytoplasm and enlarging alveoli are found, consistent with a sustained overactivity of the gland. Only a crude chemical analysis was undertaken, but the water content (Fig. 5) of the glands rises

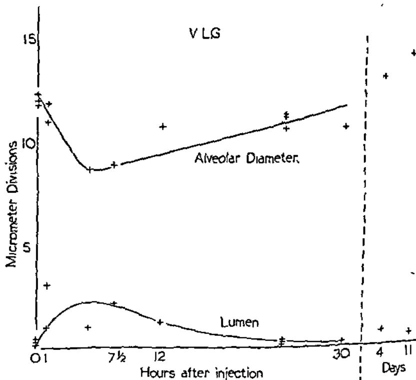


FIG 2. Diameter of alveoli and alveolar lumen in the ventral lacrymal glands after daily anterior pituitary injections.

sharply within the first day of injection, later returning to the resting value despite continued injections.

It seems clear therefore that the changes which occur under these circumstances—transient initial enophthalmos followed by sustained and massive exophthalmos—are due primarily to the fact that in guinea-pigs anterior pituitary extracts stimulate the lacrymal as well as the thyroid gland, either

through the same or different hormones. I do not want to perpetrate a dacryoadenotrophic hormone unnecessarily, since the hormone responsible appears either to be the thyrotrophic hormone or to be closely associated with it in its

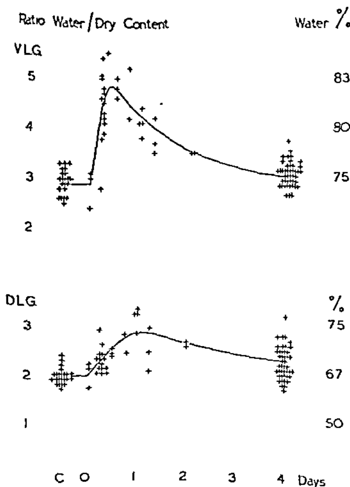


FIG 5. Water content (weight loss on drying) in relation to dry residue of ventral and dorsal lacrymal glan[...] intervals after start of daily anterior p[...] different

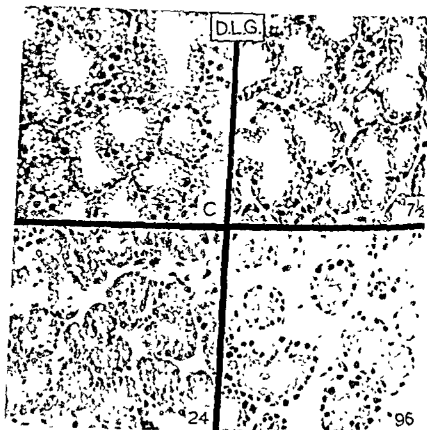


Fig 4. Dorsal, or Harderian, lacrymal glands before (C), and 7½ hours, 24 hours and 96 hours after start of daily anterior pituitary injections.

through the same or different hormones. I do not want to perpetrate a dacryoadenotrophic hormone unnecessarily, since the hormone responsible appears either to be the thyrotrophic hormone or to be closely associated with it in its

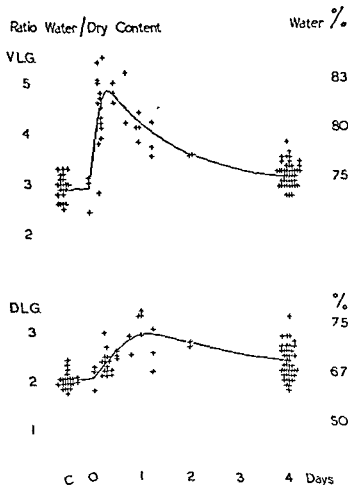


FIG. 5 Water content (weight loss on drying) in relation to dry residue of ventral and dorsal lacrymal glands excised at different intervals after start of daily anterior pituitary injections.

biological distribution. This may be tested by the method used by Chance, Rowlands and Young for other hormones, comparing the results of stimulation by extracts of pituitaries from different species. In tests of this type we have compared the dorsal lacrymal enlargement to the thyroid enlargement in the same animals after injection of pig, ox and horse pituitary extracts. With each extract the degree of lacrymal enlargement for a given thyroid enlargement has been similar and, despite the large differences in thyrotrophic hormone content of these extracts, the ratios of percentage lacrymal to percentage thyroid enlargement after four daily injections were:—

0·26 for pig pituitary, with standard error . 0·04
0·23 for ox pituitary, with standard error . 0·06
0·22 for horse pituitary, with standard error 0·05

This is evidence of biological association and not of chemical identity of the relevant hormones. It does however suggest that in these experiments in the guinea-pig, exophthalmos may have occurred because the thyrotrophic hormone stimulates the lacrymal as well as the thyroid gland.

I would like to suggest several comments upon these observations.

Firstly, this pathogenesis may not account for all forms of

although some might prove to be secondary, for examp the vascular disturbances associated with what is probably a grossly unphysiological overstimulation of the glands. This seems unlikely, in view of Smelser's work with excision of the Harderian gland and orbital implants, but needs consideration.

Secondly, are we throwing light on human exophthalmos? Lacrymal gland changes are not conspicuous in Graves's disease and man does not possess a Harderian lacrymal gland. The human condition develops slowly as compared with the guinea-pig's and these animal results seem to offer no resemblance to what is found in man.

And thirdly, if the pituitary does stimulate the lacrymal glands, as well as the mammary glands, what other exocrine glands, for example in the alimentary tract, does it stimulate? And would so crude a test as the water content of a gland a few hours after injection facilitate the detection of such actions? In fact, to what extent is the pituitary not only the conductor of the endocrine orchestra, but also the leader of the exocrine band?

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DISCUSSION

PARKES: This symposium would not have been complete without producing one new hormone, or at least a new name for a hormone, and now we've had it, together with a great quantity of experimental observation on what is one of the most baffling features of the hyperthyroid syndrome.

SMELSER: It would be a great deal more fun if Dr. Pochin and I would have a real basis for disagreement, but during the past few years our experiments have been somewhat similar and I think our results are in quite complete harmony. I am a bit envious that Dr. Pochin has seen

this gland and either put in its place some fat from other parts of the body or orbital fat from other donor animals. We were able to produce an exophthalmos without the presence of the Harderian gland. The dense mucilaginous material which accumulates in the fat and connective tissue around the Harderian gland is similar to that found in connective tissues around some other glands, and I am not certain

Pochin, and in the human when there is no actively growing gland. I am quite certain of that, but we still don't know with certainty whether the guinea pig exophthalmos is the same as, or only simulates, that which is found clinically.

Many people have the idea that exophthalmos is caused by the thyrotrophic hormone, and it may be. I believe this is basically because clinicians have associated *growth of the thyroid, thyrotoxicosis, and* exophthalmos, but I think in all of Dr. Pochin's experiments, and certainly in all of mine, and with a very few exceptions, in most of the work that has been done on this subject, crude extracts have been used. *They contain thyrotrophic activity, but one* doesn't *know that the* effect on the orbit, and the consequent exophthalmos, is due to the contained thyrotrophic hormone. It might be a "Harderian-trophic" *hormone*, as Dr Pochin suggested. Until one has worked with relatively pure *preparations I* don't *see how one can ever decide whether the* cause of exophthalmos is *thyrotrophic hormone or something* else.

In answer to Dr. Pochin's last question, I *might* suggest that the dorsal or Harderian gland isn't the only one which reacts to these anterior pituitary extracts In the lids of these animals, you *find little meibomian glands—a single pair, one in the upper lid and one in the* lower—which hypertrophy also. At the other extremity of the guinea pig, you'll find some sebaceous glands around the rectum, similar in type—one portion rather discrete and the other diffuse *They also hypertrophy at the same time as the eyes bulge out.* I don't know what kind of trophic hormones cause that. This happens in the absence of the ovaries or of the testes and in the absence of the thyroid. How can this be the "thyrotrophic *hormone*" when it's acting in the absence *of the thyroid on something quite unrelated to the thyroid* gland?

I was interested in the comment by Dr. *Folley* that the *mammary* gland was a particularly good gland for studying synthesis of fats. The Harderian gland is large, it's an exocrine gland and its secretion *is very largely fat. It would be nice if someone would get interested* in the formation of some of the lipids of this exocrine gland.

RAWSON. I would like to make a little more confusion if I may. Several years ago when Dr Albert and Dr Money were studying the effect on the eyes of the *Fundulus* of pituitary *extracts rich in* thyrotrophic hormone, they found that administration of an extract of sheep's pituitaries which they had prepared themselves, produced a rather dramatic and fairly long-lasting "popeye" in the *Fundulus*. This occurred in about 12-15 *hours and lasted for three* or *four days*. I tried to repeat their observations with some beef pituitary extracts, and couldn't We then set up the same experiment together, using the beef pituitary preparation and the sheep pituitary preparation *in other animals, and we stayed up all night observing the animals* at hourly intervals We found that the beef pituitary extract produced a minimal exophthalmos about three to four hours *after* administering the hormone, that disappeared, whereas the sheep pituitary *extract produced a rather striking* exophthalmos which lasted for three or four days. These *preparations* had about the same thyrotrophic activity. I believe that Dr. Dobyns is working with this again and is *finding* similar results

There were *other* interesting things about the *observations* made by Dr. Money and Dr. Albert. They observed that they *could remove*

from behind the orbit a milky fluid which when injected into another *Fundulus* would again produce an exophthalmos.

One of the most interesting observations was made in animals treated with thiouracil The thiouracil administered to the *Fundulus* produced a good hyperplasia without any eye changes if the water was changed every day; however, if the animals were left in the water they had originally, and if it was allowed to become contaminated with their body excreta, they did develop an exophthalmos—another observation that we could not explain.

There is another clinical observation that has intrigued me for a long time. Gynæcomastia occurs not uncommonly in male patients with Graves's disease. We've had the impression recently that this occurs to a greater degree and probably more frequently in the patients who present a more severe eye picture, than in those patients who have more classic hyperthyroidism without severe exophthalmos. I am interested in knowing whether Dr Smelser and Dr Pochin have any comments on that clinical observation.

On the other side, in support of this being a thyrotrophic factor is this, when we incubated pituitary extracts rich in thyrotrophic hormone with thyroid tissue, we found that the thyrotrophic activity is lost, and that the gonadotrophic activity is not lost, but that the exophthalmos promoting activity, as assayed in the guinea pig by Dobyns' method, is inactivated or lost following exposure to thyroid tissues.

POCHIN: With regard to the point Dr. Smelser raised about the ventral

different factors might conceivably be involved.

may induce some of these changes The second thing I wanted to suggest is that in our studies with the labelled thyrotrophic hormone preparations (which were quite inhomogeneous) we found a significant concentration of radioactivity in the extra-ocular material In other words, it's not aqueous or vitreous, and I'm not prepared to say what it is

POCHIN. We haven't excluded ACTH activity, but we failed to get any exophthalmos with posterior pituitary extracts sufficient to give good antidiuresis in the guinea pig

SMELSER. I don't think it's ACTH In recent experiments the

addition of ACTH to a preparation of pituitary which caused exophthalmos did not enhance its effect. The preparations producing exophthalmos contain very little pressor or oxytocic material, and powerful preparations of posterior pituitary don't stimulate the Harderian gland or produce exophthalmos.

MYANT: Dr. Smelser, have you ever chanced to look at the salivary glands? I think it's an interesting suggestion that the ordinary exocrine glands may be influenced, because certain glands, in man at any rate and I think in other animals too, concentrate iodide almost as much as the thyroid. The salivary glands do; the pancreas doesn't. Have you chan

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PART VI
ADRENOCORTICOTROPHIC HORMONE

ON THE BIOASSAY OF ADRENOCORTICOTROPHIC HORMONE

*CHOH HAO LI**

BEFORE any discussion of the problems of the bioassay of the adrenocorticotrophic hormone (ACTH), it may be well to summarize the physiological function of this hormone. In the absence of the adrenal glands ACTH exerts no metabolic effects (Ingle *et al.*, 1948); this means that the biological effects of ACTH are produced indirectly through its stimulation of the adrenal cortex.

Physiological Studies

Inhibition of Growth. Since Moon (1937*b*) described retardation of growth in immature gonadectomized rats after large doses of crude adrenocorticotrophic extract, evidence has accumulated to confirm the growth-inhibiting activity of ACTH. Injections with highly purified ACTH protein cause inhibition of growth in both immature and adult male rats (Evans *et al.*, 1943). The inhibiting effect of the hormone on body weight gain cannot be accounted for by differences in food intake. Furthermore, in adrenalectomized rats this effect is not seen.

*The author wishes to acknowledge the co-operation of the following

In hypophysectomized rats, the biological potency of the growth hormone is antagonized by the action of the adrenocorticotrophic hormone (Marx *et al.*, 1943). This antagonism is evident also from the osseous development (Becks, Simpson, Marx *et al.*, 1944). Recent experiments in this laboratory (Asling *et al.*, 1951) have explored further the retardation of "terminal" growth by the administration of a small dosage of ACTH in rats hypophysectomized at 21 days of age.

Studies by Baker *et al.* (1948*a*) have shown that the growth of hair and skin becomes retarded in ACTH-treated rats. There occurred a thinning of the epidermis, reduced growth of hair, an inconstant reduction in the size of the sebaceous glands, increased compactness of the dermal connective tissue, and reduction of the panniculus adiposus. These observations appear to be in harmony with the findings of Butcher and others who discovered that growth of hair is accelerated by adrenalectomy (Butcher and Richards, 1939; Butcher, 1941; Ralli and Graef, 1943).

Since protein anabolism is closely related to the process of growth, the inhibiting action of ACTH may be interpreted as resulting from anti-anabolic or catabolic effects on protein synthesis. It is therefore not surprising to note that ACTH causes a significant increase in the blood amino-acid content in normal and hypophysectomized rats (Li, Geschwind and Evans, 1949). Moreover, the hormone increases the urinary excretion of nitrogen in normal and diabetic rats (Gordon *et al.*, 1946; Bennett and Li, 1947). Recent studies of Hoberman (1950) using isotopic glycine showed that the rise in the output of nitrogen following treatment with ACTH may be the result of a more rapid rate of protein breakdown and a decrease in the rate of incorporation of amino-acids into protein.

Effects on Carbohydrate Metabolism. The production of glycosuria in normal rats by ACTH injection was first achieved by Ingle *et al.* (1946). Normal male rats were force-fed a fluid diet which represented approximately 15 grams of available carbohydrate per day. The animals developed

glycosuria on the second day following the beginning of the injection period and the condition continued as long as the hormone was given. This observation was later confirmed and extended in alloxan-induced diabetic rats (Bennett and Li, 1947). It was found that ACTH consistently produced a marked increase of the glycosuria in these rats, both with and without exogenous insulin.

The production and aggravation of glycosuria in normal and diabetic rats by ACTH treatment may be explained by the inhibition of carbohydrate utilization or the increment of gluconeogenesis, or both. Experiments of Bennett (Bennett, Applegarth and Li, 1947) appear to indicate that ACTH influences carbohydrate metabolism not only by increasing gluconeogenesis from protein but also by depressing carbohydrate utilization. This conclusion was derived from studies in diabetic rats maintained on a carbohydrate-free diet.

The fact that ACTH inhibits carbohydrate utilization could well be the result of the antagonistic action of the hormone to insulin. Recent data, obtained with the diaphragms of ACTH-treated animals, show that the rate of glucose uptake with and without insulin remains unchanged but that the insulin effect on glycogenesis is decreased. In the absence of insulin, glycogenolysis actually occurred in the diaphragms of the treated rats (Li, Kalman and Evans, 1949). From this experiment it is evident that the insulin effect in promoting glycogen storage is impaired in the diaphragms of animals treated with adrenocorticotrophic hormone.

Effects on Fat Metabolism. The liver fat of fasting normal or hypophysectomized rats has been shown to be increased by acute treatment with ACTH (Li, Simpson and Evans, 1949*a*). In normal rats force-fed a medium carbohydrate diet, treatment with ACTH for 10 days caused an increase in liver fat along with other effects (Li, Ingle *et al.*, 1949). The carcasses of hypophysectomized rats, fed a limited quantity of food and treated with ACTH, showed a gain in fat content (Li, Simpson and Evans, 1949*b*). Using

histological techniques Baker *et al.* (1948*b*) found fatty infiltration of the rat liver after ACTH treatment.

A regular increase in the amount of sudanophilic lipid in the cells of brown fat in ACTH-treated, intact and castrated rats has been reported by Baker, Ingle and Li (1950). Treatment of the hypophysectomized animal with ACTH results in an increased turnover of liver phospholipids (Geschwind *et al.*, 1950). It should also be mentioned that ACTH increases the ketonæmia and the ketonuria of fasted normal rats (Bennett *et al.*, 1948).

Effects on Electrolyte Metabolism. Since the adrenal cortex is known to affect the electrolyte metabolism it would be expected that ACTH would have a similar marked effect. It was found that there was an increased urinary excretion of potassium and phosphorus in ACTH-treated rats (Ingle *et al.*, 1946; Li, Ingle *et al.*, 1949). There were no consistent changes in the excretion of sodium and chloride in these animals; however, in human subjects the administration of ACTH caused an initial retention of the latter two elements (Luft *et al.*, 1950).

ACTH treatment did not appear to influence the uptake of radio-calcium (^{45}Ca) by the femurs of the intact rats, but did effect a significant reduction of the uptake in the hypophysectomized group (Ulrich *et al.*, 1951). No significant differences in the serum levels of ^{45}Ca were found between the treated and control groups. In normal and hypophysectomized male rats the administration of adrenocorticotrophic hormone in doses sufficient to cause hypertrophy of the adrenals produced a significant decrease in the alkaline phosphatase level of the plasma (Li *et al.*, 1946). These changes in ^{45}Ca uptake and in plasma alkaline phosphatase activity may have resulted from the effect of the hormone in retarding both chondrogenesis and œsteogenesis, as has been indicated by histological studies (Becks, Simpson, Li and Evans, 1944).

Tornblom (1949) has observed an increase in serum inorganic phosphones in ACTH-treated hypophysectomized rabbits which were kept on a phosphate-deficient diet rich in

calcium. The injection of ACTH into hypophysectomized rats restored the turnover rate of ^{32}P in all tissues (liver, kidney, adrenal and intestine) except muscle and brain (Gemzell and Samuels, 1950).

The injection of ACTH produced a significant hypoferræmia in normal rats, an effect not found after adrenalectomy (Hamilton *et al.*, 1951). On the other hand, it has been found that the administration of ACTH increases the total circulating volume of red cells in normal rats and prevents its decrease in hypophysectomized rats (Garcia *et al.*, 1951).

Effect on Lymphoid Tissues. The administration of ACTH has produced a rapid decrease in the weight of thymus and lymph nodes in the mouse and rat (Dougherty and White, 1943*a*; Li *et al.*, 1943) and an absolute lymphopenia in the mouse, rat and dog (Dougherty and White, 1943*b*; Reinhardt *et al.*, 1944). A rapid decrease in the number of lymphocytes in the thoracic duct lymph has been obtained by a single injection of the hormone (Reinhardt and Li, 1945; Yoffey *et al.*, 1946). Moreover, Yoffey and his colleagues (Yoffey *et al.*, 1951) recently observed a significant increase in the lymphocyte content of the bone marrow after the administration of a single dose of ACTH

The circulating eosinophils are strikingly depleted by ACTH injections (Forsham *et al.*, 1948). This effect was suggested as a functional test for the adrenal in man (Thorn *et al.*, 1948).

Direct Effect on Adrenal Cortex. The adrenal hypertrophy produced by ACTH has been shown to be confined mainly to the cortex. Deane and her collaborators (Deane, 1950) demonstrated that the zona glomerulosa of the adrenal cortex of the rat is not under the influence of the anterior hypophysis. When rats undergo hypophysectomy (Simpson *et al.*, 1943) the zona fasciculata is greatly reduced and [illegible]

A single injection of ACTH results in an immediate fall in adrenal ascorbic acid and cholesterol in rats and guinea-pigs

(Sayers *et al.*, 1944, 1946). It has been suggested that these observations give an indication that these substances are related to the manufacture and/or the secretion of adrenocortical hormones (Long, 1947). In young chicks ACTH causes adrenal hypertrophy without altering the ascorbic acid content of the gland (Jailer and Boas, 1950).

Survival of the Hormone in the Circulation. From the rate of disappearance, as estimated by bioassays, of a single intravenous injection of ACTH in rats, certain observations were clear. It was found that the plasma level of injected ACTH falls in a logarithmic fashion against time and that the rate of disappearance is rather rapid, the biological half-time of disappearance being calculated as 5·5 minutes (Greenspan *et al.*, 1950) By the technique of parabiosis, Van Dyke *et al.* (1950) were able to conclude that the average life of the circulating ACTH at physiological levels in the rat was approximately 17 minutes.

Bioassay in the Intact Animals

The procedure of bioassay using the 21-day-old normal male rats was first suggested by Moon (1937*a*). The amount of ACTH necessary to cause an increase in adrenal weight of 50 per cent over the controls, when injected into 21-day-old male rats for a period of three days, was defined as one normal rat unit. The data fail to fall onto a rectilinear log-dose response curve (Moon and Hansen, 1940). Because of various substances and conditions which will cause adrenal hypertrophy in the intact animal, it is clear that such assays in the intact animal are not at all specific.

Our recent data (Hayashida *et al.*, unpublished) indicate that the highly purified ACTH protein preparation gives only slight increase of adrenal weight in the Moon test. A total dose of 5 mg. caused about 10 per cent increase over the adrenal weights of the controls. When the same dose was injected as an alum-hormone complex (precipitate) a 45 per cent adrenal weight increase was achieved (see Table I). This indicates that the poor response of the highly purified ACTH

protein preparation in causing adrenal hypertrophy in 21-day-old male rats is probably due to the rapid disappearance of the hormone from the circulation. When the alum precipitate was used the increased potency which resulted was obviously the consequence of the delay in absorption.

Table I

EFFECT OF ALUM PRECIPITATE ON THE ACTH POTENCY IN 22 DAYS OLD MALE RATS

Six rats in each group.

Preparation	*Body weight*	*Adrenal*	*Thymus*
	g	*mg*	
ACTH	59 0	19 8 (16 9–25 2)†	86 2
ACTH—Alum ppt *	60 7	25 0 (22 7–28–0)	35 3
Alum-Saline	63 5	18 0 (12 8–21 8)	149 3

Injected once daily for three days, animals were autopsied on the fourth day

*To 100 ml ACTH solution (1 5 mg /ml), add 5 ml 1 per cent alum solution neutralized to pH 4 7–4 8 with 0 1 N NaOH Shake well before injection

†Ranges in parentheses

Evidence of the potency of ACTH can be adduced from the hæmatological effects of the adrenal steroids which are released when ACTH is administered. We have investigated the blood eosinophil level in unfasted mice after ACTH preparations have been injected intraperitoneally for four hours. In the controls receiving saline the eosinophil counts averaged 497 plus or minus 17 cells per cu.mm.; injections of 20 μg. of ACTH produced a fall of 17 plus or minus 5 per cent (Reinhardt *et al.*, 1951). There was a considerable variation in the fall of eosinophil levels within the range of both the control and the treated groups, so that in order to obtain really reliable determinations it would be necessary to use large numbers of animals in each group. In addition, it must be kept in mind that equivalent amounts of ACTH preparations, which have been shown by other assay techniques to possess different

adrenal-stimulating activities, may still produce equivalent eosinopenic responses.

Bioassay in the Hypophysectomized Animal

The great advantage of using hypophysectomized animals for bioassay is the absence of non-specific effects due to the cessation of the animal's own secretion of the tropic hormone; thus the effects noted are wholly resultant from the stimulation of the administered material. This increases the sensitivity and specificity of the method and makes reliable quantitative analysis possible.

Repair Test. Female rats, 26–28 days of age, were hypophysectomized and the adrenals were allowed to regress for 14 days. At this time, daily intraperitoneal injections of ACTH were given for 4 days, followed by autopsy 24 hours after the last injection. For microscopic sections the adrenals were fixed in formol, cut as frozen sections, and stained with Sudan Orange. The changes observed at the lowest effective dose levels of ACTH were as follows: individual cells constituting the zona fasciculata and glomerulosa became larger; lipid droplets were finer and more evenly distributed. Some increase in the width of the zona fasciculata and a reappearance of the zona reticularis may have occurred. This minimal response was designated as a plus 1 reaction.

The test is both sensitive and specific; on the other hand, it has a low degree of accuracy.

Maintenance Test (Simpson, Evans and Li, 1943). Hypophysectomized male rats (40 days old) were injected intraperitoneally daily (except Sunday) for 15 days (13 injections). A unit was defined as the daily dose which maintained the preoperative adrenal weight under these conditions; that is, a daily dose of approximately 0·2 mg. of pure ACTH protein isolated from sheep glands. Increasing doses of ACTH produce increments in the adrenal weight which fall into a linear log dose-response relationship. Groups of 15 to 25 animals are probably necessary, however, for satisfactory data.

The maintenance test has been considered, in general, more satisfactory than the repair test, but it has several disadvantages: a 2-week injection period is necessary, a large total amount of hormone is required and the accuracy of the determination at various dose levels is relatively poor.

It should be pointed out that in all of the preceding methods an increase in the frequency of injections greatly increases the response, presumably because of the rapidity of destruction of ACTH. The data in Table II show that the potency of the

Table II

COMPARISON OF A GIVEN DAILY DOSE OF ACTH IN MAINTENANCE TEST WHEN INJECTED ONCE OR TWICE DAILY

Daily total dose, 0·20 mg. ACTH Protein

No of daily injections	*No of rats*	*Body weight at autopsy*	*Adrenals*
		g	*mg*
0*	12	114 8	10 3±0 4†
1	11	121 4	20·6±1·3
2	7	117 1	25 9±1 6

*Control group
†Mean ± standard deviation

material in maintaining the size of the adrenal is enhanced if the same dose of ACTH is divided into two daily injections.

Bioassay by the Ascorbic Acid Depletion Method

By far the most sensitive method for the bioassay of ACTH has been estimation of adrenal ascorbic acid depletion, as standardized by Sayers *et al.* (1948). Male rats weighing 120–160 g are maintained at a constant temperature for 1 week prior to hypophysectomy. Twenty-one to 27 hours after hypophysectomy the animals are anæsthetized with sodium pentobarbital intraperitoneally, and the left adrenal is removed. The solution to be assayed is injected via the tail vein, and one hour later the right adrenal is removed. The adrenals are carefully freed of connective tissue under a dissecting lens and weighed on a double-hook-precision-weighing-balance (Roller-Smith). They are homogenized

with sand and 4 per cent trichloroacetic acid in a glass homogenizer, and the total ascorbic acid content is determined by the method of Roe and Keuther (1943).* It has been found that the ascorbic acid content of the two glands of the hypophysectomized animal does not differ by more than 25 mg./100 g. adrenal, and that the ascorbic acid depletion is a specific function of the ACTH administered. A rectilinear relationship exists between the depletion and logarithm of the dose over the range of 0·15 to 2·5 micrograms of highly purified ACTH.

The data obtained in the standardization of sheep ACTH protein are presented in Table III. From calculations of the

Table III

STANDARDIZATION OF SHEEP ACTH PROTEIN BY THE ASCORBIC ACID DEPLETION METHOD

Dose ACTH µg /100 g BW	*Depletion ascorbic acid mg per cent*[a]	*Significance of difference between means*[b]	
		Groups compared	*P value*[c]
Controls	− 6 ± 8 3 (27)		
0 2	− 43 3± 7 3 (23)	0 2 vs controls	<0 01
0 5	− 59 9± 3 2 (10)	0·5 vs 0·2	0 25
1 0	− 73 0± 6 4 (13)	1 0 vs 0 2	<0 01
		1 0 vs 0 5	0 10
2 0	−100 5± 6 3 (11)	2 0 vs 1·0	<0·01
3 0	−100 7±11 6 (12)	3 0 vs 2 0	0 50
5 0	−115 8± 6 9 (13)	5 0 vs 3 0	0 25
		5 0 vs 1 0	<0 01
10·0	−145 4± 4 8 (8)	10 0 vs 5·0	<0 01
20·0	−149 3± 9·7 (10)		
50 0	−194 8± 6 9 (5)		

a

b

c

significance of the difference between the means at each dose level, it can be shown that for groups of 10 to 12 animals an

*Dr. I. I. Geschwind in the writer's laboratory has found that the temperature of incubation in the procedure of Roe and Keuther for the determination of ascorbic acid can be increased to 57°C. and the solutions allowed to react for 45 minutes. By this modification, the whole assay procedure can be considerably shortened.

increase in dose of approximately 5 times was necessary to produce a significant increment in the response. In our hands the accuracy of the method has not been as high as that achieved by Sayers *et al.*

As shown in Table IV, there is no decrease in sensitivity to injected ACTH, as judged by adrenal ascorbic depletion, in

Table IV

EFFECT OF ACTH PREPARATIONS ON THE ASCORBIC ACID DEPLETION IN THE ADRENALS OF HYPOPHYSECTOMIZED RATS OF 1, 2 OR 3 DAYS POSTOPERATIVE

Preparation	*1-day postoperative*	*2 days postoperative*	*3-days postoperative*
L 2145 MP	−103, −118, −114, −102, −108	−105, −107, −115	−79, −93, −66
L 2214 A	−110, −132, −138		−65, −39
L 2212 A	−101, −113, −98	−96, −104, −86	
L 2210 B	−107, −130, −128	−117, −72, −94	

Dosages are at 2 μg per 100 g body weight, depletions of ascorbic acid are in mg per 100 g adrenals

hypophysectomized rats on the second postoperative day. On the third postoperative day, however, sensitivity is diminished. We have also been investigating the sensitivity of different strains of rats in response to the same dosage of ACTH preparation. So far, the three strains (Long-Evans, Sprague-Dawley, and Wistar) studied are equally reactive to ACTH administration (see Table V).

Evaluation of Adrenocorticotrophic Activity by Various Assay Techniques

The procedures for bioassay described above are based on different criteria for changes in the adrenal caused by ACTH injections and can be classified as follows: biochemical—ascorbic acid depletion in the adrenal; morphological—the repair and maintenance tests; hæmatological—eosinopenia and lymphopenia responses. Does the whole ACTH protein molecule provoke all these changes in the adrenal? Can we prepare an active ACTH peptide preparation which will provoke only one type of response? Since it has been discovered and established that the hydrolysates of the ACTH

Table V

BIOASSAY OF ACTH PREPARATIONS IN VARIOUS STRAINS OF HYPOPHYSECTOMIZED RATS BY THE ASCORBIC ACID DEPLETION METHOD

Preparation	*Long-Evans*	*Sprague-Dawley*	*Wistar*
L2062SP (Peptide)	−160, −196, −68, −101 (−131)*	−140, −146, −61 (−116)*	
L2062QP (Peptide)	−172, −177, −145 (−165)†	−118, −152, −87 (−119)†	
L1607M (Protein)	−134, −121, −101, −162, −111, −112, −103, −84 (−116)†		−156, −102, −143, −161, −80, −116 (−126)†

Values are the depletion of ascorbic acid in mg per 100 g adrenal, in parentheses are average values.

*Assay at 1 gamma *N* level.

†Assay at 5 gamma *Solid* level.

protein retain biological potency, these questions are constantly being raised in the minds of investigators.

The data to be presented in the following section are obtained from three different ACTH preparations: (1) ACTH protein prepared from sheep glands by a modified procedure previously described (Li *et al.*, 1943). (2) ACTH pepsin digest prepared by a method described earlier (Li, 1948). (3) ACTH acid digest prepared by acid hydrolysis of ACTH as follows: One gram of ACTH protein is dissolved in 90 ml. 1·0 *M* HCl and the solution is kept in a boiling water bath for 30 minutes. After cooling, 10 ml. 50 per cent trichloroacetic acid (TCA) is added. The precipitate is removed by centrifugation; to the supernatant is added 11 ml. saturated TCA solution. The precipitate formed is dissolved in water and extracted with ether to remove the remaining TCA. The aqueous solution is then frozen and dried in vacuum. The final dry powder is called ACTH acid peptide mixture (Li, 1951).

It may be seen from Table VI that the ACTH acid peptide has the highest ascorbic acid depleting potency; while the ACTH pepsin digest mixture is about twice as active as the ACTH protein, it is significantly less active ($P < 0 \cdot 01$) than the acid peptide preparation. When these preparations were

Table VI

BIOASSAY OF DIFFERENT ACTH PREPARATIONS BY ASCORBIC ACID DEPLETION METHOD

Preparation	*Dose*	*No of rats*	*Depletion of ascorbic acid*	*Estimated ACTH equivalent*
	µg		*mg /100 g adrenal*	
Protein	5	35	126 ± 4*	6
Pepsin Peptide	2	22	112 0± 5 8	4
Acid Peptide	2	9	148 0±11 1	13

*Mean ± standard deviation

ity in

as it

acid

peptide mixture the least active. On the other hand, the most active preparation in terms of thymolytic and lympholytic

Table VII

BIOASSAY OF VARIOUS ACTH PREPARATION BY THE MAINTENANCE TEST

Total dose, 5 25 mg in 10 days.

Preparation	*No of rats*	*Body weight*	*Adrenal* (1)	*Thymus*	*Cervical lymph nodes*
		g	*mg*	*mg*	*mg*
Control (Saline)	3	104	9 3 (7–10)*	268 (187–328)	91 (61–113)
Protein	5	110	16 2 (14–19)	125 (95–234)	69 (51–103)
Pepsin Peptide	5	102	13 2 (11–15)	62 (32–142)	48 (44–61)
Acid Peptide	4	107	11 2 (11–13)	207 (138–267)	122 (101–149)

*Ranges in parentheses

Table V

BIOASSAY OF ACTH PREPARATIONS IN VARIOUS STRAINS OF HYPOPHYSECTOMIZED RATS BY THE ASCORBIC ACID DEPLETION METHOD

Preparation	*Long-Evans*	*Sprague-Dawley*	*Wistar*
L2062SP (Peptide)	−160, −196, −68, −101 (−131)*	−140, −146, −61 (−116)*	
L2062QP (Peptide)	−172, −177, −145 (−165)†	−118, −152, −87 (−119)†	
L1607M (Protein)	−134, −121, −101, −162, −111, −112, −103, −84 (−116)†		−156, −102, −143, −161, −80, −116 (−126)†

Values are the depletion of ascorbic acid in mg per 100 g adrenal, in parentheses are average values.

*Assay at 1 gamma *N* level

† Assay at 5 gamma *Solid* level.

protein retain biological potency, these questions are constantly being raised in the minds of investigators.

The data to be presented in the following section are obtained from three different ACTH preparations: (1) ACTH protein prepared from sheep glands by a modified procedure previously described (Li *et al.*, 1943). (2) ACTH pepsin digest prepared by a method described earlier (Li, 1948). (3) ACTH acid digest prepared by acid hydrolysis of ACTH as follows: One gram of ACTH protein is dissolved in 90 ml. 1·0 *M* HCl and the solution is kept in a boiling water bath for 30 minutes. After cooling, 10 ml. 50 per cent trichloroacetic acid (TCA) is added. The precipitate is removed by centrifugation; to the supernatant is added 11 ml. saturated TCA solution. The precipitate formed is dissolved in water and extracted with ether to remove the remaining TCA. The aqueous solution is then frozen and dried in vacuum. The final dry powder is called ACTH acid peptide mixture (Li, 1951).

Table VIII

BIOASSAY OF ACTH PREPARATIONS BY THE MAINTENANCE TEST AND ASCORBIC ACID DEPLETION METHOD

Preparation	Maintenance test				Ascorbic acid depletion method		
	Daily dose	No. of rats	Body weight	Adrenal	Dose	Depletion of ascorbic acid	Estimated ACTH equivalent
	mg		g	mg	μg	mg/100 g adrenal	μg
L1607M (Protein)	0·2	30	129·2	23·7±0·7*	5	−134, −121, −101, −72, −84, −158, −138, −162, −111, −112, −103, −84, −60, −124, −104, −104, −117, 130 95 (−111·3)†	3·9
L1622A (Protein)	0·2	42	130·7	24·5±0·8	2	−150, −128, −52, −62, −107 (−100)	2·5
L1660CS (Peptide)	0·2	11	130·6	19·0±1·3	5	−99, −115, −128, −77 (−104·7)	3·1
L1563DI (Peptide)	0·2	18	108·3	20·3±1·7	2	−68, −105, −80, −143, −107, −134 (−106·5)	3·3

*Mean ± standard deviation
†Average values in parentheses

activity, but with only intermediate adrenal weight maintenance effect, was the ACTH pepsin digest. Furthermore, the data of Table VIII show that the ACTH pepsin peptide is significantly less active in maintaining adrenal weight than the protein preparation.

It should also be noted in Table VII that the acid peptide exhibiting the highest ascorbic acid depleting potency is the least active in causing reduction in weights of thymus and cervical lymph nodes. The suggestion must be made that high ascorbic acid depleting potency is not necessarily related directly to the process by which the adrenal secretes substances causing reduction in thymus and lymph node weight (Reinhardt and Li, 1951). This conclusion is further supported by the studies of Hungerford, Reinhardt and Li (Hungerford *et al.*, 1952), investigating the effect of various ACTH preparations on the lymphocyte content of thoracic duct lymph in the rat. Results, summarized in Table IX, give clear indication that the ACTH peptide is unable to effect reduction of the lymphocyte counts, whereas administration of ACTH protein produces a rapid decrease in the number of lymphocytes in the thoracic duct lymph.

The findings represented by these data suggest at least one consideration and several long-term questions. For the time being, the data indicate the necessity for making parallel assays of ACTH preparations (protein and peptides) by at least two methods, until such time as we can establish and define more clearly the correlation between experimental observations on the one hand, and activity as determined by various biochemical and morphological assay techniques. One possibility for the future which will obviate these difficulties is that when components of the ACTH protein are eventually isolated through various chemical procedures, the result may be a purified peptide preparation which will exhibit activity in only one or the other of the various bioassay methods. Some further questions which must be raised to clarify the whole problem are (*a*) the possible presence in the ACTH preparation of two or more active

factors, (*b*) the effects which are consequent upon differences in rate of absorption, utilization, and excretion, and (*c*) the rôle of trace contaminants.

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Table IX

EFFECT OF DIFFERENT ACTH PREPARATIONS ON THE FLOW RATE AND TOTAL CELL CONTENT OF THORACIC DUCT LYMPH OF 60-DAY-OLD MALE RATS

Mean values presented with standard errors in parentheses.

Preparation	*Dose*	*No of rats*	*Body weight*	*WBC*	*Per 24 hours*		*Per 100 g /24 hours*	
					Lymph flow	*Total cells*	*Lymph flow*	*Total cells*
	mg		*g*	*per cu mm*	*ml*	*millions*	*ml*	*millions*
Control		28	252	31500	21·0 (0·8)	642 (38)	8·4 (0·3)	232 (13)
0·9 per cent NaCl	0·5	10	240	22600	23·8 (2·3)	321 (47)	9·9 (0·9)	215 (13)
ACTH Protein	3·0	13	240	21600	14·8 (0·7)	317 (20)	6·2 (0·3)	134 (12)
ACTH Pepsin Peptide	1·5	10	238	19200	17·4 (1·3)	322 (34)	7·3 (0·5)	135 (14)
ACTH Acid Peptide	1·5	14	233	28900	20·7	614	8·9 (0·5)	262 (30)

All injections made intraperitoneally two hours prior to lymph collection

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DISCUSSION

LONG: We require a precise method of assay, as the present ones all leave much to be desired. What is your experience with the cholesterol [illegible] there are after three hours definite differences between them.

SONENBERG: What are the criteria of homogeneity of your ACTH preparations?

LI: It would take the whole morning to answer that question. Preparations A and B appear to be different by chromatography. These were very crude preparations

PRUNTY: You found that the acid peptide is more active in causing a depletion of ascorbic acid and less active in producing a fall in eosinophils?

LI: We also found that the acid peptide preparation is very poor in the adrenal maintenance test

[illegible] speaking only as an old standardizer—what we need to know is that different batches of ACTH are standardized with regard to their therapeutic effects. The clinicians need a method which will enable

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OVARIAN RESPONSE TO ACTH

*F. T. G. PRUNTY and BARBARA E. CLAYTON**

In the past there has accumulated much evidence, based on embryological, experimental and clinical studies, suggesting an endocrinological relationship between the adrenal cortex and the gonads. Many aspects of this subject were excellently reviewed recently by our Chairman (Parkes, 1945). It is possible to extract from the work that has been done certain apparent guiding principles, and it is worth remembering that the adrenal cortex and gonad are derived from adjacent areas of the genital ridge.

Some animals show cyclical changes in the adrenals associated with the reproductive cycle. This appears to be particularly marked in the rat, where cortical enlargement accompanies œstrus (Bourne and Zuckerman, 1941). In the mouse the situation appears more complex, though it seems certain that the X-zone regresses with sexual activity and in pregnancy (Howard-Miller, 1927; Deanesly, 1928).

On the other hand, increased adrenal cortical activity would appear to diminish certain phases of ovarian function. Thus, amenorrhœa is found to occur in Cushing's syndrome and the adrenogenital syndrome. With the advent of cortisone and ACTH it has been noticed that these hormones sometimes induce amenorrhœa when used for therapeutic purposes (Hench *et al.*, 1950). The administration of cortisone interrupts pregnancy in mice (Robson and Sharaf, 1951).

Under certain circumstances there are indications that the ovaries can exert an adrenal cortical-like activity. Survival after adrenalectomy is prolonged in various species, including rodents, by pregnancy (Firor and Grollman, 1933), administration of progesterone (Pfeiffer and Hooker, 1940), and treatment

*Holden Research Fellow.

ACTH to be used as an alternative to cortisone. Which of the present methods can be regarded as indicating a "cortisonogenic" activity?

LI: We have used the ascorbic acid test extensively.

DALE: You measured the potency of your fractional preparations by the ascorbic acid depletion test, because the test is easy to perform, and gives fairly reproducible results. But these might be definitely misleading with regard to the therapeutic efficiency of different samples

LONG: When you measure the effect on white blood cells you are measuring the cortisone released from the gland by ACTH.

DALE: How is such an organization as the National Institute of Medical Research to standardize batches of ACTH for therapeutic potency?

LONG. Up to now there has not been any systematic assessment of ACTH activity using all the methods now in use on the same preparations.

DALE: From the practical view any of the methods now in use may be misleading. If they place the same preparations in different orders, they cannot all be right.

LI: Some tests are more popular than others, but a great deal more work on the methods of assay needs to be done.

requirement for cortical replacement became relatively large after delivery by Cæsarian section. On another occasion we observed marked periorbital œdema at the commencement of the menses in a patient with Addison's disease implanted with the maximum tolerated dose of DCA.

That the ovaries have some controlling influence on the activity of the adrenal cortical cells has been beautifully demonstrated by Woolley (1950). Certain strains of mice are prone to develop nodular cortical hyperplasia or adrenal cortical carcinomata after gonadectomy.

For several years Albright and his colleagues (Albright *et al.*, 1942) have considered that there is a pituitary hormone, in their opinion probably luteinizing hormone, which exerts an effect on both ovary and adrenal cortex. With regard to the latter gland the function of this hormone has been thought to be mainly stimulation of androgen production. In their view, the occurrence of hirsutism in certain women with cystic ovaries is due to stimulation of luteinizing hormone in association with the arrested ovarian follicular phase. On the other hand, the work of Hamblen and his colleagues (1941) would seem to show that a rise in 17-ketosteroid excretion accompanies total ovarian lack and ovariectomy. This is more in accord with evidence produced by Smith (1946) indicating increased adrenal cortical function with regard to electrolyte and carbohydrate metabolism in the ovariectomized rat, and the experiments reported here by Dr. Nelson, in which chorionic gonadotrophin failed to produce increased 17-ketosteroid excretion in castrate men. However, it is widely held that ovarian œstrogen secretion is an important factor in the stimulation of pituitary luteinizing hormone production. It is also generally agreed that œstrogen administration in the rat produces adrenal cortical enlargement in the presence of the pituitary. The significance of this latter fact is complicated by the extremely toxic effects of œstrogen in adrenalectomized animals noted by a number of workers, including Pfeiffer and Hooker (1940), and in the course of experiments in our laboratory.

with pregnant mares' serum extract (Pfeiffer and Hooker, 1940). The experiments demonstrating these facts strongly suggest that survival after adrenalectomy is correlated with the presence of a corpus luteum. Pseudopregnancy is also effective, so that it seems unlikely that the fœtuses are concerned. Swingle *et al.* (1937) showed that adrenalectomized bitches do not require cortical hormone during pseudopregnancy.

A seasonal variation in the survival after adrenalectomy has been found. For example, Bülbring (1937), observed that the amount of cortin required to maintain adrenalectomized drakes varied with the time of year, and she found a correlation between the amount of cortin required and testicular size. Parkes (1945) suggests that after the breeding season the testes might elaborate substances that supplemented corticosterone.

The grafting of ovaries into the ears of adrenalectomized mice assists in the maintenance of life (Hill, 1948). Using an ACTH preparation with lactogenic activity, but containing no gonadotrophin, thyrotrophin, or growth hormone, Davidson and Moon (1936) were able to show prostatic and seminal vesicle stimulation in castrate mice.

Patients with Addison's disease may improve, possibly due to placental activity, during the latter part of pregnancy, once the strain of morning sickness has terminated. Thorn *et al.* (1942) have commented on this and have drawn attention to the very critical period immediately following delivery. Much of the literature has been reviewed by Knowlton *et al.* (1949) In two of their patients they observed significant rises in 17-ketosteroids as pregnancy advanced, but suggested they might be of placental origin. An improvement in blood pressure was noted during the latter part of pregnancy in a patient with Addison's disease observed by us. She was thirty at the time of the event, and had eight years' history of the disease. Up to the sixth month there was a steady fall in blood pressure which then underwent spontaneous reversal. The patient remained exceptionally well throughout the pregnancy without any increase in therapy. The

The results obtained following adrenalectomy appeared to indicate a seasonal change. During the second half of November 1950 it was found that even though the adrenals were absent ACTH still produced inhibition. In December 1950 and the first half of January 1951 only some adrenalectomized mice showed inhibition, and by the second half of January 1951 none showed complete inhibition.

Complete data on uterine weight and ovarian size of 21-day-old mice in the colony have been available every week for eighteen months. The uteri have constantly weighed 2–3 mg. except for an interval in the early part of the winter when they have weighed 7–10 mg. and occasionally more. The ovaries associated with these large uteri showed follicles in which antrum formation appeared to be more prominent. During this time, too, the uteri are unduly but irregularly responsive to urinary follicle-stimulating hormone.

After a lull in breeding during September and early October these mice were the first offspring produced during the new breeding season. The 21-day-old mice maturing prematurely were litter-mates of the 6–9 weeks old adrenalectomized mice in which ACTH could cause inhibition of healing, and the correlation is shown in Fig. 1. These findings of an apparent seasonal variation suggested that a gonadotrophin might be playing a part. Accordingly, a further series of experiments were performed as shown in Table II. Thus, mice born at the beginning of the new breeding season, pregnant mice, and those pre-treated with chorionic gonadotrophin showed inhibition, even when adrenalectomized.

Cortisone will cause inhibition in the gonadectomized mouse. It seems likely that the gonads, particularly under the influence of luteinizing hormone, are capable of producing one or more unknown compounds which will cause inhibition. Other than cortisone, of the steroids examined, only progesterone produced a slight effect in a dosage of 2·5 mg. per animal

A further interesting experiment seemed to indicate an action by ACTH in the absence of the adrenals. ACTH or

This, then, was the general position when our experiments now to be described were performed. They seem to indicate that the mouse ovary has an adrenal cortical-like action provided it is in the luteal phase (Clayton and Prunty, 1951*b*).

The indication used of supposed adrenal activity was the inhibition of experimental wound healing in mice. The details of the technique have been fully described elsewhere (Clayton and Prunty, 1951*a*, *b*). Under the experimental conditions used, the 50 per cent inhibiting dose for any group of mice was 45 μg. ACTH (H7911, potency = 6 × La-1-A), and 2 mg. of cortisone acetate. The percentage of animals failing to heal in any group is proportional to the log of the dose of ACTH given, and by using probits the potency of various preparations of ACTH can be compared.

Experiments showed that pitressin tannate and gonadotrophin would not inhibit healing, and that the sample of ACTH used did not contain any gonadotrophin. Operative interference of any kind causes some impairment of healing, but by careful control of all experiments this did not prove a disadvantage.

The preliminary experiments carried out, and the results obtained are summarized in Table I.

Table I

Mouse	*Effect of ACTH on healing*
1. Intact male and female mice	Inhibition.
2. Hypophysectomized male and female mice	Inhibition at one day. No inhibition at eight days.
3. Ovariectomized mice	No inhibition
4. Ovariectomized and adrenalectomized mice	No inhibition.
5. Gonadectomized male mice.	No inhibition
6. Gonadectomized and adrenalectomized male mice.	No inhibition
7. Adrenalectomized male and female mice.	Variable response, probably seasonal. At the beginning of the breeding season—inhibition. Later—*no* inhibition.

receiving daily ACTH gained in weight, and the scrota, vulvæ, and dependent parts of their faces became swollen and œdematous.

Conclusion

These experiments seem to indicate that under certain conditions, particularly when luteinization is induced, the mouse ovary is able to respond to ACTH with a cortisone-like action causing inhibition of wound healing. The question of the nature of the hormone concerned is an interesting speculation. It would appear unlikely that endogenous progesterone only is concerned for two reasons:—

(*a*) Chorionic gonadotrophin alone proved insufficient stimulus to prevent healing.

(*b*) Administration of progesterone produced poor response, whereas Pfeiffer and Hooker (1940) reported that one-fifth of the dose used would maintain life in adrenalectomized mice. It remains to be seen whether further evidence is forthcoming to suggest elaboration of a C_{21} ketonic steroid by the ovary.

Acknowledgements

We would like to thank Miss J. Hammant and Mr. A. E. Clark for their invaluable technical assistance during these experiments.

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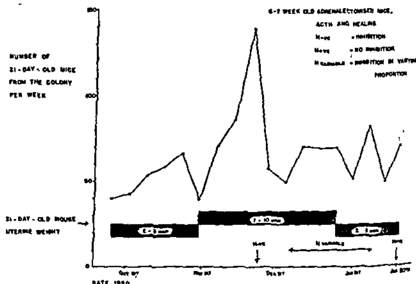


FIG. 1. Correlation between seasonal breeding, uterine weight, and healing response to ACTH.

saline was administered daily to groups of 21-day-old male and female mice in February 1951 for 10 days. It was found that whereas untreated, or saline treated, adrenalectomized mice usually lost weight or remained stationary, those

Table II

Mouse	*Effect of ACTH on healing*
8. Pregnant adrenalectomized mice.	Partial or complete inhibition, becoming more marked as pregnancy proceeds. This effect rapidly disappears postpartum
9. Adrenalectomized female mice, after 2 weeks pretreatment with postmenopausal FSH or PMS.	No inhibition.
10. Adrenalectomized female mice, after 2 weeks pretreatment with human chorionic gonadotrophin.	Inhibition.

LONG: Jailer, I think, suggested that the placenta might produce ACTH or something like it.

CLAYTON: If males are given gonadotrophin over a long period their wounds do not heal so well as in controls.

BROBECK. Why was there no inhibition when the adrenals were intact?

CLAYTON: We should like to know.

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DISCUSSION

HUME: I am not clear about the methods used to assess wound healing Was this judged by the presence of granulation tissue?

PRUNTY: Yes

HUME: In my opinion, this is not necessarily a good criterion—on ACTH wound healing can occur without the normal amount of granulation tissue. I think "healing" is not an accurate term to use in this connection.

CLAYTON: If any change is found in the wound it is taken to indicate healing. If there were hyperæmia, for instance, we would say that there was no inhibition.

HUME: Is this after 24 hours?

CLAYTON 28 hours.

BUSH: How long before the experiment were the animals ovariectomized?

PRUNTY: Two days.

TUCHMANN-DUPLESSIS Might the seasonal changes be due to the influence of the thyroid?

PRUNTY. The animals in the second season behaved in precisely the same way as in the first season We have not had the opportunity of looking into the thyroid side of it yet

HOUSSAY: Dr. Pourneau Delille has published in the *Presse Médicale* on the formation of granulation tissue In adrenalectomized rats granulation formation is very exaggerated, especially if they are spayed females.

LI: I am not clear about what Dr. Prunty obtained with his ACTH. Does it have a direct effect on the ovaries?

PRUNTY: Yes.

[illegible] and [illegible]

on the testis.

CLAYTON: Were the ovaries pre-treated with luteinizing hormone?

LI: No. You assume that ACTH is synergistic to gonadotrophin?

CLAYTON: It seems so.

hypophysectomized animals. The dosage is expressed in mg. The therapeutic effect of *Cortiphyson* on various diseases has been acknowledged by Lohmeyer, Husselmann and Bansi (1950) in Hamburg, and Heilmeyer (1951) and is equivalent to foreign preparations.

In our clinic *Cortiphyson* was used for the first time in a case of chronic arthritis. It was a child of 11 whose illness had so far not been influenced by any other therapy. With a daily dosage of 45–90 mg. a noticeable improvement of the illness could be observed. During ACTH treatment a drop of the eosinophil counts, an increase in the fasting blood sugar and a decrease in the erythrocyte sedimentation rate could be seen. Alterations of nitrogen and electrolyte metabolism did not vary from those in adults.

We then tried to find out the effect of *Cortiphyson* on healthy thriving infants, using a lower dosage of 7·5–22 5 mg. per day. *It was interesting to notice that in each case the normal* growth was impeded to a great extent during ACTH treatment. *Cortiphyson* therefore seemed to have a "dystrophying influence" on the healthy thriving child.

As you see in the first figure, the daily increase of weight was diminished and sometimes stopped completely. In some cases we even noticed a decrease of weight during ACTH treatment. We cannot explain this fact as a consequence of alterations in water and mineral metabolism, for the intake of food and fluids had not been changed and the daily amount of urine and chlorides in the urine was not increased. On the other hand, estimation of the nitrogen excretion revealed an increase until the nitrogen balance ran negative. At the same time the uric acid and the creatinine excretion was raised. Therefore we assume that ACTH affects the growing tendency of infants by its strong influence on the protein metabolism.

As an example I should like to show you in the second diagram the effect of ACTH on the nitrogen balance and creatinine excretion of an infant· the balance runs negative, the creatinine excretion rises in spite of an unchanged diet. Obviously, this is of much greater importance to an infant

EXPERIENCES IN THE ADMINISTRATION OF ACTH TO INFANTS

E. ROMINGER

In the course of our investigations on the pathogenesis of severe nutritional disturbances in infancy, which we call in Germany "Säuglings-Dystrophie," we have tried to elucidate the effect of different hormones on the metabolism of the infant. Recently we have gathered some experience in the use of cortisone and, particularly, ACTH.

Under the term "Säuglings-Dystrophie" we understand a severe disturbance of nutrition in infancy. The children do not thrive normally in spite of optimum feeding, and develop severe marasmus even when there is no evident abnormality of digestion, such as diarrhœa. In fact, these infants starve for "endogenous reasons." The principal cause of this severe illness is not yet known. It is quite possible that the disturbance of growth results from insufficient anabolism of tissue, probably in consequence of inadequate utilization of the proteins fed. According to the results of new investigations, we believe that hormonal factors, mainly ACTH, are involved in this process.

In order to learn something about the part which ACTH is taking in the pathogenesis of infantile dystrophy, we first had to watch the effect on healthy thriving infants. In doing so we made some remarkable observations concerning the effect of ACTH on the growing organism, and these, I think, are of general interest. Therefore I should like to give you a short report on our investigations.

The ACTH we used is named *Cortiphyson*, a preparation of the firm Promonta—Hamburg. *Cortiphyson* is at present the only available ACTH preparation in Germany. It is sta

Apart from investigating the protein metabolism, we tried to learn something about the effect of ACTH on carbohydrate metabolism in infants. For this purpose we carried out dextrose tolerance tests by giving 3 grams of glucose per kilogram body weight orally or 0·3 grams intravenously, and we watched the fasting blood sugar and the reaction of the blood sugar after each injection of ACTH. Thereby we made the following observation: during ACTH treatment up to ten days, the glucose tolerance tests showed a diminution of tolerance to

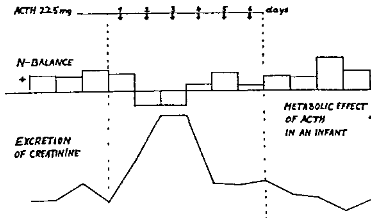


FIG 2 Metabolic effects of ACTH Negative N-balance and increased excretion of creatinine during ACTH treatment.

glucose in one single case only. This diabetic curve became normal again within a few days after the treatment with ACTH had been stopped In no case could glycosuria be detected. The increase in the fasting blood sugar reported by some authors was not seen in our infants.

After each injection of ACTH, however, we noticed a considerable increase of the blood sugar; the average increase was about 80 per cent of the initial level. I have not been able to find out the exact explanation for this fact.

Furthermore we were specially interested in the hæmatological effects of ACTH in infants. We continuously

than to an adult, as the infant grows and can only do so as long as the nitrogen balance keeps positive.

The anti-anabolic effect of ACTH was already presumed in adults. Looking at the weight chart and the nitrogen balance

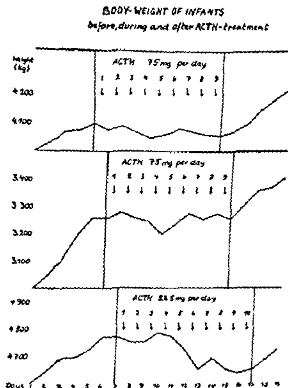


FIG. 1. Stationary or decreasing weight during ACTH treatment.

we can show impressively that even during a short period of treatment with ACTH the growth of infants is retarded. Comparing the effects of other hormones with those of ACTH we come to the conclusion that ACTH indeed has an antagonistic effect to the somatotrophic hormone of Li and Evans (1945), as has been previously suggested.

in the urine. There remains only one explanation: the adrenal cortex of the infant does function, but apparently in a way which varies a little from that of the adult. Probably the lack of eosinopenia after ACTH treatment is a sign of incomplete activity of compound F in infants.

The effect of ACTH on the infant, however, is in many ways similar to that of compound E, i.e. cortisone. In fact, we have been able to create a temporary retarding of the growth of infants by using cortisone acetate. Even with a dosage of up to 50 mg. of cortisone per day a regular decrease of eosinophils in the peripheral blood could not be achieved.

Comparing cortisone and ACTH with each other, we found that we required a much larger dose of cortisone—about double the amount—to get the same effects as we did with ACTH.

In our experience the control of lymphocytes and leucocytes proved to be a much more useful test of the effect of the use of ACTH on infants than the eosinophils. Our clinical observations have shown clearly that ACTH, when given in sufficient dose, retards the growth of a healthy infant even within a few days. The antagonistic effect of ACTH to the somatotrophic hormone of Li and Evans (1945) can be shown most convincingly in infancy.

The importance of this fact regarding the pathogenesis of disturbed growth in infancy has still to be investigated.

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DISCUSSION

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controlled the peripheral blood and the bone marrow of our children with the following results: after each injection of a sufficient amount of ACTH the lymphocytes dropped within two hours to almost 50 per cent: simultaneously the neutrophil leucocytes increased in numbers until they reached a peak after about 4 hours. This probably means that the neutrophil leucocytes are being mobilized. If you draw the curves of lymphocytes and neutrophils synchronously one underneath the other, it is seen that the two types of cells reach their maximum reaction at different times. You get the impression that the two hæmatological systems react independently from each other.

The reticulocytes on the other hand show no alteration nor do the red cells or the hæmoglobin values. Contrary to the observation of Koller (1950), we have not been able to establish an increase of thrombocytes when treating our infants with fully effective doses of ACTH.

In the bone marrow we have seen a regular decrease of the mature granulocytes which can only be interpreted as a consequence of an increased output of these cells. At the same time we notice a relative increase of immature granulocytes, i.e. a shift to the left.

To my mind it is remarkable that the lymphatic cells in the marrow are almost unchanged in number although they are reduced significantly in the peripheral blood. Therefore I think we are allowed to conclude that the changes in the lymphocytes of the peripheral blood take place without the corresponding reaction in the bone marrow. Yet there has been no proof for the suggestion that the decrease of lymphocytes under ACTH treatment is caused by their destruction.

We have not been able to estimate the effect of ACTH injections by only controlling the eosinophils. Although the number of eosinophils is occasionally reduced, this cannot be considered a rule. But one cannot conclude from this that the [illegible]

SOME REMARKS ON THE PATHOPHYSIOLOGY AND THERAPY OF THE ADRENOGENITAL SYNDROME

A. JORES, H. NOWAKOWSKI and F. RAUSCH

In the course of the past few months we have had occasion to make a number of new observations in three cases of congenital adrenogenital syndrome in children.

The first case was that of a 13 years old girl with all of the clinical signs of interrenalism. Determination of the 17-ketosteroids in the urine showed an excretion of 30 mg. daily, with normal values for the beta-fraction. To exclude an adrenal tumour a perirenal insufflation was made, the result of which suggested an adrenal hyperplasia. Since an adrenal tumour could not be excluded with certainty and an atresia of the vagina also suggested the possibility of zygotic hermaphroditism, an exploratory laparotomy was performed, which confirmed our tentative diagnosis of feminine pseudohermaphroditism with adrenal cortical hyperplasia. Thus far, the case history of our patient presents nothing remarkable. The postoperative course and lethal termination, however, made it interesting and instructive.

Following the operation, which presented nothing of note, an intestinal atonia ensued, which, however, could be quickly controlled. On the third day after the operation, vomiting commenced. Emesis was continuous, and marked circulatory weakness followed, so that the surgeon considered the possibility of an Addisonian-crisis, a condition observed occasionally in young pseudo-hermaphrodites. A saline infusion was made and 4 ml. of adrenal cortical extract were *injected immediately following the infusion.* A few minutes after this injection there was a completely unexpected attack

counts were done two hours and four hours after the injection and were counted in a counting chamber.

HUME. An absolute count? I can't understand why there was no response unless the total dose was too small. In my opinion intramuscular ACTH should be given four times a day for maximum effect.

ROMINGER: The counts in my investigations were absolute counts According to the Thorn test the eosinophils should have shown a [illegible]

studied over a period of six months and given 8 mg. a day in four divided doses we found no inhibition of growth up to 30 days, slight inhibitory effects over 30 days, and significant inhibition after three months.

LONG: What was the age of these rats?

LI: We gave them ACTH from day 1.

LONG: There is a recent report from the Merck Institute of marked inhibition of growth after cortisone.

LI: Those were young hypophysectomized rats injected from the day of operation, and with continued injections for 30 days. The inhibition of growth was quite obvious.

COURRIER: I have also seen inhibition with cortisone. I injected pregnant rats and I found that the litters were smaller than in the controls, and there was marked atrophy of the adrenals.

SMELSER: ACTH and cortisone are used in ophthalmology to reduce growth of retrolental tissue in infants with retrolental fibroplasia; inhibition of body growth and infection are limiting factors.

PRUNTY: Professor Rominger, in one of the charts you put up the nitrogen balance curves [illegible] of seven, but the weight curves w[illegible] nt. Have you tried to corr[illegible] ges? Were your urinary specimens complete?

ROMINGER: I am sure that we got hold of the complete daily urinary excretion. Besides, the variations of the nitrogen balance from day to day are already considerable under normal conditions. Therefore I didn't correlate the nitrogen balance with the daily weight changes. On the other hand, we think we can draw the conclusion from the negative nitrogen balances after some days of ACTH injection that in [illegible] seen instantly.

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of tonic-clonic spasms, foaming at the mouth and a rise in temperature to 41°C. In spite of all measures taken, the child died of circulatory failure. The autopsy confirmed our clinical diagnosis.* The hypertrophy of the clitoris was very obvious. Vagina and urethra ended in the common urogenital sinus. The internal genitalia, uterus and ovaries, were hypoplastic; in their development, however, they corresponded to the age of the patient. There was no indication of testicular tissue. Both [illegible]
skull findings [illegible]
were exaggera[illegible]
part of the skull was thin and atrophied. The sutures of the skull were partially obliterated. The brain itself† showed a remarkable increase in weight. It weighed 1,300 g.—in a child of 13 years—whereas the average weight of the adult brain is 1,250 g. The convolutions of the cortex were flat and there was a definite pressure conus of the cerebellum. On frontal sections the ventricles were found to be *narrow*, especially in the region of the anterior and lateral horns, as well as the aqueduct. These findings are indicative of generalized brain œdema or swelling. There were no signs of brain tumour.

Summarizing the clinical and pathological findings, the cause of death in this case was an acute œdema of the brain following the operation. The pronounced digital impressions and the atrophy of the skull are indicative of increased intracranial pressure of longer duration.

The question arises why increased intracranial pressure of chronic nature and, finally, an acute œdema of the brain developed. It is well known, that increased production of steroid hormones influences skeletal growth profoundly. There is in the adrenogenital syndrome a premature calcification of the epiphyseal cartilages, but this is also the case in

*The autopsy was performed in the Institute of Pathology of the University of Hamburg (director: Prof. C. Krauspe). An extensive report on the pathological findings is in preparation.

†We are indebted to Prof. H. Spatz, Giessen, for his invaluable advice and help.

other parts of the skeleton, for instance in the cartilages of the ribs.

It seems plausible to assume that the ossification of the skull sutures in the adrenogenital syndrome takes place before the determination of normal brain growth, physiologically ending in the third decade of life. Premature craniostosis naturally leads to a cessation of growth of the skull. Since the brain continues to grow, however, a disproportion between skull volume and brain volume must necessarily ensue. We are familiar with this condition in the case of turricephaly. So far as we know no similar observations in the adrenogenital syndrome have been published before. The neurosurgeon is familiar with the fact that chronic intracranial pressure favours development of acute œdema of the brain. Slight stress, such as operation, infection, hydræmia, etc., may suffice to produce a catastrophic reaction with lethal end. In our case we must assume that the trauma of operation, infusion of NaCl and adrenal cortical extract produced the conditions leading to brain œdema.

With regard to the question, whether it is possible to influence androgen overproduction pharmacologically, we present clinical data from two other patients. Two sisters, aged 10 and 13½ years, presented the same symptoms, differing only in the degree of development. The younger girl was still in her phase of growth acceleration (the roentgenograms showed open epiphyseal sutures). She was already as large as her older sister. The latter had stopped growing 2 years before due to premature closure of the epiphyseal sutures. There was also a marked difference in the somatic type of the two girls; the younger was slender, girlish, whereas the other represented a more boyish type with strong muscle development. The axillary and pubic hair was equally developed in both. In the elder sister there was an indication of a beard. Regarding the external genitalia a marked hypertrophy of the clitoris was obvious. The external and internal genitalia were of female type; urethra and vagina separate and gynæcological examination showed the development of

of tonic-clonic spasms, foaming at the mouth and a rise in temperature to 41°C. In spite of all measures taken, the child died of circulatory failure. The autopsy confirmed our clinical diagnosis.* The hypertrophy of the clitoris was very obvious. Vagina and urethra ended in the common urogenital sinus. The internal genitalia, uterus and ovaries, were hypoplastic; in their development, however, they corresponded to the age of the patient. There was no indication of testicular tissue. Both adrenals were appreciably hypertrophied. The skull findings were especially conspicuous: digital impressions were exaggerated on the scalp and the osseous tissue in this part of the skull was thin and atrophied. The sutures of the skull were partially obliterated. The brain itself† showed a remarkable increase in weight. It weighed 1,300 g.—in a child of 13 years—whereas the average weight of the adult brain is 1,250 g. The convolutions of the cortex were flat and there was a definite pressure conus of the cerebellum. On frontal sections the ventricles were found to be narrow, especially in the region of the anterior and lateral horns, as well as the aqueduct. These findings are indicative of generalized brain œdema or swelling. There were no signs of brain tumour.

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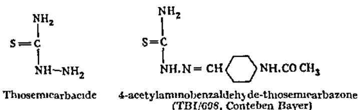
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(Fig. 2) shows the action of these substances on the excretion of the 17-ketosteroids, cortin fraction and the plasma colloids determined by electrophoresis. The values of the various protein-fractions are represented in percentage values. The dosage is indicated at the top.

In the case of the elder girl, in whom the average excretion of 17-ketosteroids, as already mentioned, amounted to 70 mg., the excretion dropped gradually after treatment to an average value of 30 mg. daily The reduction of 17-ketosteroids is significant and certainly beyond the physiological range of variation. In spite of increasing dosage of thiosemicarbazone



Thiosemicarbacide 4-acetylaminobenzaldehyde-thiosemicarbazone (TBI/698, Conteben Bayer)

FIG 1

it was impossible to reduce the 17-ketosteroid values further. Immediately following discontinuation of the drug, 17-ketosteroid excretion rose again Entirely different were the levels of the cortin and the alcoholic fraction. Here, a clear-cut increase could be observed. Especially interesting were the values for plasma colloids: there was a decrease of alpha-2 and gamma-globulins, as several authors have stressed (Herrnring and Kuhlmann, 1951). The shift in the albumin-globulin-quotient and drop in sedimentation rate are important indications of the action of thiosemicarbazone Whether the plasma-colloid shift precedes the drop in the 17-ketosteroids or occurs simultaneously, is still uncertain.

In the case of the younger sister there was *no* significant change in 17-ketosteroid excretion in spite of the same dosage. The cortin fraction, alcoholic fraction and plasma colloids, on the other hand, showed the same trend as in the case of the elder sister.

uterus and ovaries. The question of whether we were dealing with a hormonal or zygotic form of intersexuality was decided by an assay of the 17-ketosteroids in the urine. In the younger the excretion amounted to 25–30 mg., in the elder 70 mg. in 24 hours on the average. The beta-fraction was not increased in either of the two. Roentgenograms of the adrenals in the elder showed a marked bilateral increase in size.

On the basis of the clinical picture, analysis of hormone excretion and the roentgenograms, there was no doubt as to the diagnosis of an adrenogenital syndrome with adrenal hyperplasia in both cases. Also, we made a determination of the FSH and found 6·6 MU in the younger and 24·4 MU in the elder. The cortin-fraction, determined according to the method of Heard and Sobel was rather high, on the average 2 and 3·5 mg. respectively in 24 hours.

The results of operative treatment of the adrenogenital syndrome with adrenal hyperplasia are not very encouraging. On the one hand, the mortality is comparatively high, on the other it is seldom possible to modify the clinical picture significantly even by removal of the greater portions of the hypertrophied adrenals. Wilkins (1951) has reported an excellent result with cortisone in the adrenogenital syndrome. He was able to reduce the high excretion of androgens in the urine to normal levels with comparatively small doses of cortisone. But cortisone was not available to us.

Stimulated by the observations of Heilmeyer (1950) and Kuhlmann (1951), who found a reduction in 17-ketosteroid excretion under the effect of thiosemicarbazone, it seemed to us justifiable to test the action of this substance in our cases. We considered it all the more permissible to try these substances since new derivatives of much lower toxicity than the usual ommercial products were available.

The basic substance of these new compounds is thiosemicarbazide (Fig. 1). *p*-Acetylamidobenzaldehyde thiosemicarbazone, discovered by Behnisch, Mietzsch and Schmidt (1948) is known for its tuberculostatic action (Domagk). The commercial name is *Conteben* (TB I/698). The graph

(Fig. 2) shows the action of these substances on the excretion of the 17-ketosteroids, cortin fraction and the plasma colloids determined by electrophoresis. The values of the various protein-fractions are represented in percentage values. The dosage is indicated at the top.

In the case of the elder girl, in whom the average excretion of 17-ketosteroids, as already mentioned, amounted to 70 mg., the excretion dropped gradually after treatment to an average value of 30 mg. daily. The reduction of 17-ketosteroids is significant and certainly beyond the physiological range of variation. In spite of increasing dosage of thiosemicarbazone

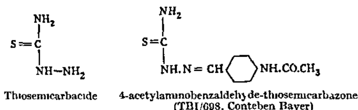


FIG. 1

it was impossible to reduce the 17-ketosteroid values further. Immediately following discontinuation of the drug, 17-ketosteroid excretion rose again. Entirely different were the levels of the cortin and the alcoholic fraction. Here, a clear-cut increase could be observed. Especially interesting were the values for plasma colloids: there was a decrease of alpha-2 and gamma-globulins, as several authors have stressed (Herrnring and Kuhlmann, 1951). The shift in the albumin-globulin-quotient and drop in sedimentation rate are important indications of the action of thiosemicarbazone. Whether the plasma-colloid shift precedes the drop in the 17-ketosteroids or occurs simultaneously, is still uncertain.

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Summarizing, we can state that there was a reduction in the 17-ketosteroid excretion of more than 50 per cent in the case of the elder sister. Excretion rose again immediately after

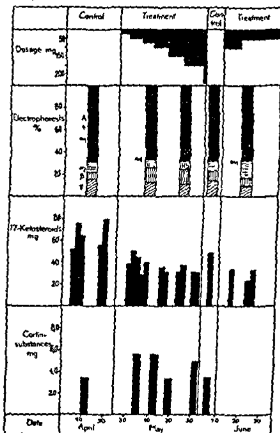


FIG. 2. Effects of thiosemicarbazone on the plasma colloids, 17-ketosteroids and cortin-substances in a 13½-year-old female pseudohermaphrodite.

discontinuation of treatment. In the younger sister, treated in the same manner, there was no decrease in 17-ketosteroid urine level. The cortin fraction and alcoholic fraction rose in both cases. Parallel to or preceding the fall in 17-keto-

steroids (as judged by findings in the elder sister) there were characteristic changes in the electrophoretic pattern. An increase in dosage over 5 g. was impossible, due to signs of early hepatic damage.

An interpretation of our findings is difficult. Besides their tuberculostatic action, the thiosemicarbazones possess other non-specific actions, as shown by a decrease in 17-ketosteroids, drop in the sedimentation rate and shift in plasma colloids. Since thiosemicarbazone derivates also are effective in rheumatoid arthritis, there was an inclination to attribute a cortisone-like action to these substances.

Regarding the mechanism of action the following possibilities have suggested themselves:—

These substances may act upon the adrenals, either indirectly by blocking ACTH secretion of the pituitary or directly by inactivation of hormone synthesis in the adrenal cortex; there may be an increased destruction of steroid hormones by the liver, which, as is well known, plays a central rôle in steroid metabolism.

In comparing our results with the findings of Wilkins after cortisone treatment, we see that cortisone reduces the excretion of the 17-ketosteroids as well as that of the cortin fraction, whereas in thiosemicarbazone treatment these two entities are influenced in opposite directions. In this respect, thiosemicarbazone and cortisone are very different in their action. The profound difference in the action of the two substances in tuberculosis we need mention only briefly. Whether the thiosemicarbazones directly influence hormone synthesis in the adrenal cortex (in view of their chemical similarity with thiouracils one could visualize this) or whether their carbazide group inactivates the ketone group cannot be decided by our observations. We know from clinical experience that a damaged liver catabolizes androgens to a higher degree and that 17-ketosteroid excretion is reduced in this process. Liver cirrhosis is an example of this. Numerous authors have described hepatic lesions due to treatment with thiosemicarbazones. The question arises, whether a depression in

17-ketosteroid excretion as observed by other authors and ourselves may not be explained on the basis of disturbed hepatic function. If this explanation for the non-specific action of the thiosemicarbazones is accepted, other findings, *such as plasma protein* changes, are easily understood. As yet, definite conclusions are not possible. Further studies are under way.

We are indebted to Dr. G. Herrnring for *performing* the *hormone* assays and electrophoretic analysis.

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[illegible]

11, 1.

DISCUSSION

SIMPSON: I would like to comment on your terminology. It is unusual to apply the term "pseudohermaphroditism" to cases not obvious before puberty

PEARSE: Was the pituitary examined, and were any changes seen in the first case? Having regard to the mode of action of the thiosemicarbazones, surely the new drug would need to be split before it could act as a blocking agent.

JORES. The results of microscopic examination were not ready when I left. Nothing was seen macroscopically. I agree with you about the drug.

GROSS: In experiments by Meier and Schuler, rats treated with [illegible] showed an increase in [illegible] l animals. If [illegible] the increase [illegible]

Cushing's syndrome. There was a normal amount of Compound F (despite the stress of operation) but four to five times as much of an abnormal steroid, probably a C_{19} steroid. In the Cushing's case, in which there was mild hirsutism, the adrenal venous blood showed large amounts of Compound F only, and none of the abnormal steroid.

A PROPOSED MECHANISM FOR THE SYNTHESIS OF STEROIDS IN THE ADRENAL CORTEX

C. J. O. R. MORRIS

DURING the past few years a considerable amount of evidence has accumulated on the synthesis of adrenocortical steroids under the influence of ACTH. The pioneer work of Long, Sayers and their collaborators has demonstrated that among the earliest effects of ACTH on the adrenal cortex is a depletion of the cholesterol and ascorbic acid content of the latter, and that almost simultaneously an outpouring of glucocorticoids occurs, as manifested by the depletion of liver glycogen. There appears to be no direct evidence that the adrenocortical steroids are in fact formed from cholesterol, although we do know that the organism is capable of carrying out all the separate stages involved. Thus Bloch (1945) has shown that labelled cholesterol can be converted to labelled pregnanediol in the human, showing that the side chain of the C_{27} sterol can be shortened to give a C_{21} steroid. The conversion of a Δ^5 3-alcohol to a Δ^4 3-ketone is known to occur *in vivo*, while the perfusion experiments of Hechter *et al.* (1950) have demonstrated that the adrenal cortex can introduce an 11-oxygen function into a suitable steroid molecule. The mechanism of 17-hydroxylation is less certain, although the co-existence of corticosterone and 17-hydroxycorticosterone as the main adrenocortical hormones in many species suggests that the adrenal cortex can also carry out this process.

The assumption will therefore be made here that cholesterol is in fact the precursor of the adrenocortical steroids.

It has been known for some time that the adrenocortical steroids are divided chemically into two groups: the C_{19} type which give rise to urinary 17-ketosteroids; and the C_{21} type,

the metabolites of which are many and varied, and indeed from a quantitative standpoint are mostly unknown.

The conversion of C_{21} to C_{19} steroids and the elimination of the latter as 17-ketosteroids has for a long time been believed to be a major metabolic pathway of the C_{21} group. This has been the basis of the clinical use of 17-ketosteroid determinations as a measure for adrenocortical function. Additional evidence for this belief appeared to be provided by the isolation from urine of 17-ketosteroids with an 11-oxygen function. However the demonstration by Hechter that the adrenal cortex *in vitro* can oxidize in the 11 position both C_{19} and C_{21} steroids now makes this evidence of little value. It is even possible that the adrenal cortex may introduce an 11-oxygen atom into steroids of extra-adrenal origin which may be presented to it via the adren l circulation.

The availability of cortisone has made it possible to examine the conversion of a C_{21} steroid into C_{19} metabolites in human subjects with greatly depressed endogenous production of adrenocortical steroids. This of course has been done by many workers, notably by Dobriner and co-workers, but I should like to present here some data on a previously untreated patient with fairly severe Addison's disease

The first figure shows the effect of ACTH at a dosage of 100 mg. daily. It will be seen that there are no significant changes in electrolyte excretion, eosinophil count or 17-ketosteroid excretion during the experimental period.* It may be inferred that the patient's adrenals were virtually incapable of responding to an ACTH stimulus.

The second figure gives the response of the same patient to 100 mg. of cortisone daily. It shows a rise in 17-ketosteroid excretion from about 4 mg./48 hours to about 10 mg./48 hours which was not sustained after cessation of dosage. The eosinophil count also shows a depletion, showing that the mechanism for this process can still respond to cortisone and together with the first figure, confirming the patient's lack of

*The marked increase in Na^+ during the 1st and 2nd 48 hr periods was due to an alteration in Na^+ intake in the diet

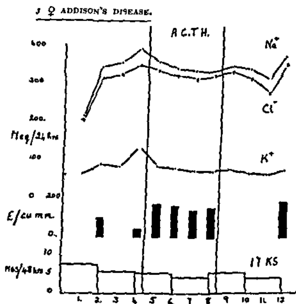


Fig. 1 Action of ACTH on an untreated case of Addison's Disease.

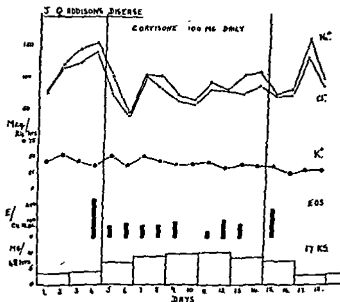


Fig. 2. Action of cortisone on the care of Addison's Disease

production of corticoids. Eosinophil counts in the period immediately before the first control period were 155–238/c.mm.

This study can be regarded as a control observation on the one which follows and suggests that conversion of C_{21} to C_{19} steroids is not a major metabolic pathway, at least in adrenal deficiency.

The third figure gives metabolic data on a pseudohermaphrodite girl of 6 years who was admitted with symptoms of

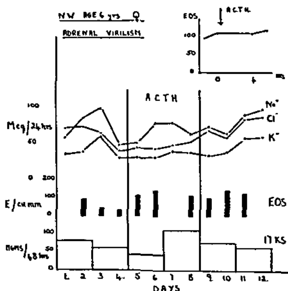


FIG. 3. Action of ACTH on a female pseudo-hermaphrodite.

adrenal virilism. ACTH was administered at a dose of 50 mg. daily. It will be seen that although there were virtually no changes in electrolyte excretion or eosinophil count, the 17-ketosteroid excretion, which was already running at the exceptionally high level of 60–80 mg./48 hours, almost doubled during ACTH administration and returned to its *former level on cessation of dosage.* This increase is statistically significant· $P > 0 \cdot 05$.

The fourth figure illustrates the response of this case t[o] cortisone acetate at a level of 100 mg./24 hours. It will b[e] seen that the 17-ketosteroid excretion declined from an initia[l] value of 33 mg./48 hours to about 6 mg./48 hours, whic[h] is almost normal for a girl of this age. The low level wa[s] maintained for some time after cessation of dosage.

The eosinophil count was depressed by the cortisone treat- ment, indicating that endogenous production of corticoids

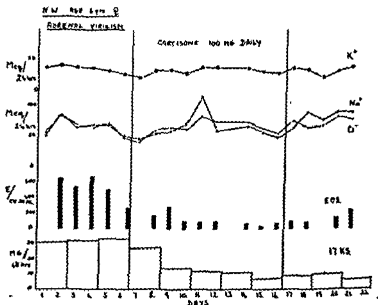


Fig 4 Action of cortisone on the female pseudo-hermaphrodite.

should have been detected if it had occurred in response to ACTH.

The conclusions which may be drawn from this case are as follows:—

(1) Endogenous production of corticoids, already at a low level, was not influenced by ACTH.

(2) Excretion of 17-ketosteroids, already at a very high level, was practically doubled by ACTH and reduced to

almost normal levels by cortisone, presumably by the direct action of the latter on the pituitary, in depressing the release of ACTH.

(3) The production of 17-ketosteroids and corticoids is brought about by separate mechanisms, only one of which, the 17-ketosteroid production, was in this case under ACTH control.

(4) It is interesting to observe that 1 ml. of this patient's urine before ACTH treatment, injected into hypophysectomized rats, brought about a 20 per cent depletion of adrenal ascorbic acid. Although the author believes that use of the Sayers ACTH assay on biological fluids is not yet on a satisfactory basis, this finding would at least suggest that ACTH production was at a high level, since the inhibitory effect of the endogenous corticoids was lacking. The high rate of ACTH output would thus account for the very high 17-ketosteroid production, since there is no evidence that substances of this type can have an inhibitory effect on the pituitary. After the cortisone treatment, the urine was negative in the Sayers test.

(5) Since the 17-ketosteroids and eosinophils responded to cortisone, the primary effect in such cases of adrenal virilism would appear to lie in some stage of the synthesis of corticoids from cholesterol The defect in many such cases is probably genetic. As the feed-back effect of the endogenous corticoids is lacking, the pituitary will turn out ACTH at a high rate and the adrenals will respond by turning out C_{19} steroids at a correspondingly high rate, giving rise to the clinical symptoms of virilism. In contrast, in Addison's disease, synthesis of both C and C [illegible]

with the very variable 17-ketosteroid outputs in such cases.

A tentative scheme which embodies these conclusions is given in Fig. 5.

The degradation of adrenal cholesterol to precursors of the C_{19} and C_{21} groups is under ACTH control and presumably

The fourth figure illustrates the response of this case to cortisone acetate at a level of 100 mg./24 hours. It will be seen that the 17-ketosteroid excretion declined from an initial value of 83 mg./48 hours to about 6 mg./48 hours, which is almost normal for a girl of this age. The low level was maintained for some time after cessation of dosage.

The eosinophil count was depressed by the cortisone treatment, indicating that endogenous production of corticoids

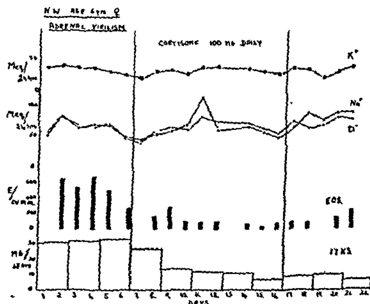


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It is tempting to compare the degradation with the chromic acid oxidation of cholesterol familiar to the organic chemist, where both C_{19} and C_{21} steroids are formed simultaneously. Although it is dangerous to force this analogy too far, it does at least indicate the weak places in the cholesterol side chain. The subsequent production of the typical corticoids from the C_{21} precursors by introduction of the Δ^4 3-ketone group, the 11-oxygen function and in some cases the 17-hydroxyl group are not under pituitary control, as is demonstrated by the *in vitro* experiments of Hechter. The order given is not necessarily the correct one, although it is likely that 17-hydroxylation is a late stage

It is hoped that the outlined scheme, though at present tentative, will serve the purpose of all good hypotheses, to provoke discussion and stimulate experiment.

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DISCUSSION

LONG These experiments of Morris, together with those of Pincus, may indicate the site of action of ACTH. Some C_2 compound seems to be the precursor of adrenal steroids, but the formation of cholesterol is not under ACTH control. Hypophysectomized animals after depletion of the adrenal cholesterol by ACTH can resynthesize adrenal cholesterol. Pincus has suggested the importance of progesterone as an intermediary in the formation of adrenal steroids but so far has not located any step that appears to require the presence of ACTH for such conversion.

FOLLEY I would like to ask Dr Long about the evidence that the adrenal can synthesize cholesterol

LONG. Chaikoff showed that slices of beef adrenal cortex will convert labelled acetate into cholesterol. Bloch showed that pregnant women excrete labelled pregnanediol after administration of labelled cholesterol

MORRIS Bloch's experiments are fairly water-tight, and as good as such experiments can hope to be. The same results are found in several species.

LONG Bloch repeated his experiments with double labelling and confirmed his original findings.

the mechanism in some way involves ascorbic acid. As we have seen, pathways A and B may be independent although it seems more likely that defects in corticoid production may

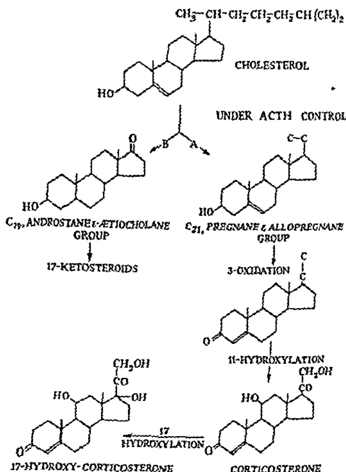


Fig. 5. Proposed scheme of synthesis of adrenocortical steroids.

be due to defects in the later stages; 17- or 11-hydroxylation, etc. From this viewpoint the C_{19} and C_{21} precursors are formed simultaneously from cholesterol and the primary function of ACTH is to induce and/or facilitate this change.

PART VII

LACTOGENIC AND MAMMOGENIC HORMONES

SOME ASPECTS OF THE PHYSIOLOGY OF THE ANTERIOR-PITUITARY LACTOGENIC HORMONE

S. J. FOLLEY

The existence of a lactation-initiating hormone of the anterior pituitary was discovered by Stricker and Grueter (1928). It is now one of the best characterized protein-hormones of the anterior lobe and is known variously as lactogenic hormone, prolactin, luteotrophin, or mammotrophin. I propose in this communication to use the term "prolactin."

Though some progress in the chemical purification of prolactin soon followed on the pioneer observations of Stricker and Grueter, there is little doubt that advance in this field was greatly facilitated by the perspicacious characterization by Riddle, Bates and Dykshorn (1933) of prolactin as the hormone present in anterior-pituitary (A.P.) extracts which causes enlargement of, and secretion in, the crop glands of the pigeon For this discovery provided the basis of a very convenient and useful bioassay method for prolactin which, in the "micro" form first developed by Lyons and Page (1935), has been widely used by those engaged on chemical work with this hormone.

In the present connection it may be noted that prolactin was the first A.P. protein-hormone to be prepared as a pure protein as judged by currently accepted physico-chemical criteria. In fact, it is probable that as long ago as 1937 Lyons had obtained almost pure preparations of prolactin,

PARKES: How did you collect your 24-hour samples?

MORRIS: They were collected by competent people, and each one was checked by the creatinine content. We've wanted to repeat these tests, but the clinicians are not keen on them.

QUERIDO: If my impression from one of your slides is correct you did not see a rise in 17-ketosteroid excretion until the third day of ACTH administration. It is our experience that after giving adequate amounts of ACTH, response is already seen on the second day.

MORRIS: We have a great deal of data, more than I presented today, especially in regard to the earlier periods, before administration of ACTH. Many of the variations that people report are due to the techniques used in the determination of 17-ketosteroids. But I feel confident of our technique. The increase is statistically significant.

QUERIDO: Our data are certainly also correct; they are run in duplicate and collections are made under strict supervision. In the first case of Addison's disease, would you regard the figure of 8 mg. for ketosteroid excretion as within the normal range?

MORRIS. I am not competent to say.

SIMPSON. There is evidence that androgens inhibit the secretion of pituitary ACTH.

MORRIS: We were estimating 17-ketosteroids and not testosterone.

SIMPSON. Have you any views on the inhibition of the secretion of ACTH by testosterone?

MORRIS. No, it's outside my province.

SIMPSON: Selye's group showed atrophy of the adrenal cortex in rats following testosterone. Clinically testosterone has been used in Cushing's syndrome to reverse or modify a negative nitrogen balance.

BUSH: According to Zaffaroni, Bloch's experiments may not be so water-tight as has been suggested. Using labelled acetate or labelled cholesterol he was able to show that Compound F from dog adrenals showed the same specific activity with either precursor. It does seem that there is a fairly direct route of synthesis from cholesterol to the cortical steroids, but at high rates of synthesis the route from acetate itself may be equally important. The evidence for the slowness and lateness of the 17-hydroxylation is reinforced by Pincus's perfusion experiments, where in single perfusion the ratio of corticosterone to Compound F was roughly constant at all levels seen. Although there may be other considerations *in vivo*, Pincus found that if the effluent from the perfused adrenal was passed repeatedly through the gland the proportion of Compound F increased steadily.

In the Cushing's case which I mentioned earlier, blood levels of Compound F were five to six times the normal, although the 17-ketosteroid excretion was only at the high range of normal and the elevation not very significant.

MORRIS: The essential point is the demonstration of the independence of the two mechanisms, and the cases in which these can be demonstrated are very rare; one in four to five years so far as we are concerned.

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these and other considerations which I cannot go into now, which led F. G. Young and myself (Folley and Young, 1941), in a critical discussion of the status of prolactin as a lactogenic hormone, to prefer the concept of lactogenesis as being a response to the co-ordinated action of a complex of A.P. hormones, centring round prolactin as an essential element, to the more commonly accepted view (e.g. Bergman and Turner, 1940) that prolactin is the sole specific lactogenic hormone. Of the other known A.P. hormones, there is reason to believe that ACTH must also be concerned in lactogenesis, since, for example, it has been found that purified prolactin will initiate lactation in hypophysectomized guinea-pigs provided they are also given adrenal cortex extract or cortisone (Nelson, Gaunt and Schweizer, 1943) It should be emphasized that prolactin is undoubtedly the limiting factor in lactogenesis in most experimental situations, though in the pseudo-pregnant rat, in which Reece (1939) was best able to initiate lactation if adrenal cortex extract was administered in addition to prolactin, the ACTH-adrenal mechanism appears to be a rather critical factor as well.

In view of the afore-mentioned striking demonstration by Lyons (1942) of localized lactogenic responses to mammary intraductal injection of purified prolactin in the rabbit, which has since been confirmed by Meites and Turner (1948), we thought it of interest to see if prolactin, *in vitro*, would bring about some characteristic changes in the respiratory metabolism of mammary gland slices, taken from rats at the end of pregnancy, which we have shown to accompany the initiation of milk secretion. In the experiments of Lyons, prolactin would appear to be the sole limiting factor for lactogenesis, and despite the findings of Reece (1939), just mentioned, it seemed worth while to begin by examining the effect of prolactin alone.

In a study of the respiration of rat mammary gland slices during various functional phases (Folley and French, 1949) we have found that tissue taken at the end of pregnancy, which exhibits a rather low endogenous Q_{O_2}, does not respond

since later preparations made by a procedure which was essentially his have been found by the pigeon crop test to exhibit the maximal potency so far found for the pure hormone (30–35 i.u./mg.), and to be homogenous as judged by solubility and electrophoretic studies. Until fairly recently more was known about the chemistry of prolactin than about that of any other hormone of the anterior pituitary. As an example of the extent of this knowledge it may be mentioned that Li (1949) has reported a complete amino-acid analysis of the hormone, accounting for all of the nitrogen.

Prolactin as a Lactogenic Hormone

Prolactin under appropriate circumstances initiates lactation in functionally competent mammary tissue, and the experiments of Lyons (1942), in which minute quantities of the purified hormone were directly injected into the galactophores of the rabbit mammary gland, thereby initiating lactation in the injected sectors but not in neighbouring ones, show that its action on the mammary epithelium is direct. However, it should be noted that Lyons used rabbits with intact hypophyses, and the co-operation of endogenous A.P. hormones in these "local" lactation responses was therefore not excluded It seems probable that endogenous A.P. hormones must indeed have played a part in these responses, since, as far as is known, purified prolactin, systemically injected, does not initiate lactation in hypophysectomized animals. Whether it will do so when locally injected into the mammæ of freshly hypophysectomized rabbits possessing suitably developed mammary glands has, so far as I know, not yet been put to the test. Systemically injected unfractionated A.P. extracts, on the other hand, have been regularly found to initiate lactation in the absence of the hypophysis.

Now, if we insist on the usual criterion for specific biological responses to A.P hormones, namely that these should be limited to responses elicited in hypophysectomized animals, there would appear to be a logical obstacle to regarding prolactin as a lactogenic hormone in its own right. It was

among which prolactin must be prominent and perhaps a limiting factor. Accordingly, Miss J. H. Balmain, in our laboratory, has investigated the effect of purified prolactin, added *in vitro*, on the total gas exchange of rat mammary gland slices metabolizing acetate+glucose, the overall pressure changes being measured in Warburg vessels, the buffer being Krebs-bicarbonate-saline in equilibrium with 95 per cent oxygen and 5 per cent CO_2. Under these conditions, if the R.Q.=1, the pressure will slowly fall as acetate is consumed and CO_2 is accordingly absorbed from the gas phase by the buffer. If, however, the slices are in a secretory state and thus exhibit an R.Q. sensibly greater than unity, the gas pressure progressively increases with time due to the excess of respiratory CO_2 over oxygen consumed. This technique has proved useful for investigating the stimulatory effect of insulin, *in vitro*, on fat synthesis by mammary gland slices (Balmain, French and Folley, 1950).

So far, Miss Balmain has been unable to demonstrate by this technique any *in vitro* effect of prolactin on the respiration of mammary gland slices taken from rats on the 20th day of pregnancy. However, in five experiments on slices from rats at days 1–4 of lactation, addition of purified prolactin caused a definite increase in the rate of net gas evolution indicating that the hormone was increasing the R.Q. of the slices. A typical result is illustrated in Fig. 2. However, it must be noted that so far we have been unable to detect any increase in the R.Q. of the slices, due to the action of prolactin *in vitro*, by the method of Dickens and Simer (1931).

In these preliminary experiments, therefore, we have evidence of lactogenic responses to prolactin *in vitro* in tissue taken early in lactation, but tissue taken at the end of pregnancy has proved unresponsive. This may indicate, as suggested by the results of Reece (1939), that in the rat at the end of pregnancy, the ACTH-adrenal cortex mechanism is a critical factor, while soon after parturition the tissue is saturated with corticoids but does not yet have its full quota of prolactin. Experiments which we plan to carry out on the

to the addition of glucose and the R.Q. is <1 whether or not glucose is present in the medium. Soon after parturition, however, the tissue actively utilizes glucose as shown by an increase of Q_{O_2} in glucose above endogenous values and the

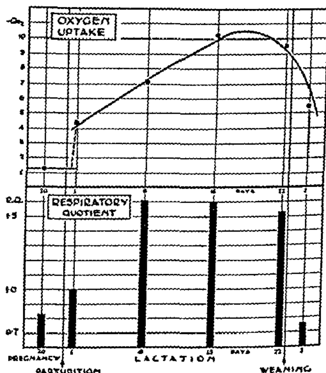


Fig. 1. Respiration in glucose of rat mammary gland slices in late pregnancy, during lactation and after weaning. Results taken from Folley and French (1949).

R.Q. (in glucose) rises above unity, attaining a value of over 1·5 at the height of lactation. These findings are illustrated in Fig. 1, which shows results taken from Folley and French (1949). The increase in Q_{O_2} (in glucose) and the rise in R.Q. above unity which begins soon after parturition would appear to be a manifestation of the lactogenic effect of A.P. hormones,

among which prolactin must be prominent and perhaps a limiting factor. Accordingly, Miss J. H. Balmain, in our laboratory, has investigated the effect of purified prolactin, added *in vitro*, on the total gas exchange of rat mammary gland slices metabolizing acetate+glucose, the overall pressure changes being measured in Warburg vessels, the buffer being Krebs-bicarbonate-saline in equilibrium with 95 per cent oxygen and 5 per cent CO_2. Under these conditions, if the R.Q.=1, the pressure will slowly fall as acetate is consumed and CO_2 is accordingly absorbed from the gas phase by the buffer. If, however, the slices are in a secretory state and thus exhibit an R.Q. sensibly greater than unity, the gas pressure progressively increases with time due to the excess of respiratory CO_2 over oxygen consumed. This technique has proved useful for investigating the stimulatory effect of insulin, *in vitro*, on fat synthesis by mammary gland slices (Balmain, French and Folley, 1950).

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effect of prolactin + cortisone *in vitro* on mammary slices might throw more light on this question.

Prolactin and Galactopoiesis

As purified prolactin preparations became available, it was

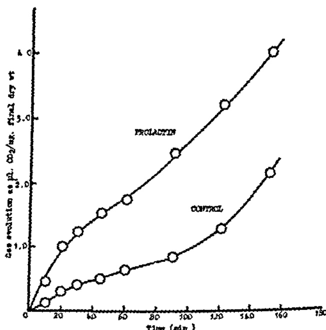


Fig. 2. Unpublished results of J. H. Balmain and S. J. Folley [illegible]

hoped that this hormone would prove useful for treating hypogalactia in parturient women, and also for increasing the milk yield of cows in the period of slow decline which normally follows the peak of lactation. Such hopes, however, have not been realized.

Many years ago, Folley and Young (1938) showed that unfractionated extracts of ox anterior pituitary were far more active in increasing the milk yield of cattle in the declining phase (galactopoietic effect) than could be accounted for by their prolactin content, and, in fact, numerous experiments carried out by us over a number of years have indicated that purified prolactin, at any rate in single injection tests, has very little, if any, galactopoietic action in the intact cow. Table I,

Table I

GALACTOPOIETIC EFFECTS OF PURE ANTERIOR-PITUITARY HORMONES MEASURED IN GROUPS OF LACTATING COWS

The results are taken from Cotes, Crichton, Folley and Young (1949).

Treatment (single injection)	*Mean increase in milk yield for two days after treatment expressed as a percentage of the yield over the two days preceding treatment*	
	Absolute	*Minus control*
Control	$-1\cdot89\pm1$ 52	
15 mg Growth hormone	2 78 ± 4 15	6 30 ± 5 87
30 mg. Growth hormone	*$6\cdot31\pm1\cdot52$	*$8\cdot20\pm2$ 15
60 mg Growth hormone	3 42 ± 2 14	6 68 ± 3 03
40 mg Prolactin	-2 34 ± 2 50	0 82 ± 3 53
10 ml. Unfractionated ox anterior-pituitary extract	*6 60 ± 2 22	*9 $90\pm3\cdot11$

*Significant at 5 0 per cent level

containing data taken from Cotes, Crichton, Folley and Young (1949), which gives some of the results of our most recent experiments carried out on groups of lactating cows, shows that a single injection of 40 mg. of pure prolactin prepared by a method essentially that of Li, Simpson and Evans (1942), and assaying approximately 30 i.u./mg., had no detectable effect on milk yield. This same table shows that on the other hand purified A.P. growth hormone was markedly galactopoietic under these conditions, and it would appear that most, if not all, of the galactopoietic activity shown by unfractionated ox A.P. extracts in single injection tests, can be accounted for by their content of growth hormone. This finding has since been confirmed by Donker and Petersen (1951).

Prolactin as a Mammogenic Hormone

Quite soon after the discovery of the lactogenic effect of A.P. extracts, some workers held that A.P. preparations containing prolactin, not only initiated lactation, but also evoked mammary growth. Unfractionated ox A.P. extracts undoubtedly exhibit mammogenic effects, as shown in a number of laboratories including our own (Cowie and Folley, 1947), but C. W. Turner and his school would undoubtedly attribute such action mainly, if not solely, to the presence of specific "mammogenic" hormones, which they claim are distinct from the six well-known A.P. hormones, but the existence of which is not yet generally accepted.

However, Lyons has always insisted that prolactin will evoke mammary growth and, in discussing his intraduct injection experiments already referred to, has stated that the purified prolactin used in these experiments caused mammary hyperplasia in addition to local initiation of lactation. Additional evidence exists that prolactin may be concerned in growth as well as secretion of the mammary gland. Thus Gardner and White (1941) have shown that the mammary gland of the hypophysectomized mouse, usually regarded as unresponsive to the growth stimulus of steroid hormones, can be made to grow by the injection of prolactin together with ovarian steroids.

Control of Prolactin Secretion

There is considerable evidence that the secretion of prolactin by the anterior pituitary can be evoked by the stimulus of suckling acting through a neural, or more probably a neurohormonal, pathway. As far as I know, the essentials of this concept were first advanced by Selye (1934) as a result of experiments in which he showed that application of the suckling stimulus to certain glands of a rat or mouse will retard involution in other glands in the same animal the suckling of which is experimentally prevented by occlusion of the teats or from which no milk can escape because of ligation of the galactophores. This striking retardation of

involution, coupled with the maintenance of a secretory condition in the epithelium, Selye attributed to reflex release of prolactin by the anterior pituitary in response to the suckling stimulus. It was later shown by Hooker and Williams (1941) that the mammary involution which so swiftly follows weaning, can be similarly postponed by injections of prolactin. Since the original work of Selye, a considerable body of evidence has been accumulated, all of which supports the concept that the secretion of prolactin is in certain circumstances closely related to the suckling stimulus. Though the nervous pathways involved have not yet been worked out, it would seem possible by analogy with the work of de Groot and Harris (1950) on the control of ACTH release by the anterior lobe, that suckling reflexly excites a centre in the hypothalamus which in turn, by a humoral mechanism in which the hypophyseal portal system is involved, evokes the secretion of prolactin by the anterior lobe.

On the other hand, the rôle of œstrogen in prolactin secretion must be taken into consideration. Meites and Turner (1942) have carried out numerous experiments on the prolactin content of the rodent pituitary which led them to advance a theory to explain the initiation of lactation at parturition and its inhibition during pregnancy. Briefly, their theory states that high titres of circulating œstrogen following parturition evoke the release of prolactin from the anterior pituitary, thus initiating lactation, this effect of œstrogen being nullified during pregnancy by the action of progesterone. An important piece of evidence cited in support of this theory consists in the demonstration that the total pituitary prolactin content is increased by œstrogen treatment whatever the dosage, but not if sufficient progesterone is administered as well. I have no time now to go into technical points concerning the validity of this work and the techniques used; these have been critically discussed elsewhere (Folley and Malpress, 1948) and further work with the object of meeting some of these criticisms has been published (Meites and Turner, 1948).

One important criticism may however be pointed out now, namely that an increased pituitary prolactin content may indicate inhibition of release (i.e. increased storage) rather than increased secretion. Meites and Turner (1948) however cite one experiment in which it was claimed that œstrogen caused a rise in blood prolactin levels. If this result were confirmed beyond doubt this particular criticism would be disposed of.

In the present connection, however, whatever view one takes of the validity of arguments based on changes in the pituitary prolactin content of the laboratory rodent, note must be taken of the undoubted fact (see review by Malpress, 1947) that œstrogen in low levels exerts a lactogenic effect *pari passu* with a mammary growth stimulating action in farm animals, secretion beginning before any milking stimulus is applied. This has recently been strikingly demonstrated in experiments in our laboratory which have so far only been reported in preliminary form (Cowie, Folley, Malpress and Richardson, 1951).* In ovariectomized goats receiving 0·25 mg. hexœstrol/day, the artificially grown udders soon showed secretion and became so distended that they had to be milked well before the end of the 20-weeks' treatment period. Animals receiving higher doses of œstrogen alone, or progesterone (40 or 100 mg./day) in addition to 0·25 mg. hexœstrol/day, showed good udder growth but no evidence of secretion until the treatment was stopped and milking begun. In some respects these results fit in with the concept of Meites and Turner; in others they are in harmony with the "double threshold" theory of Folley and Malpress (1947), which postulates two thresholds of anterior-pituitary response to circulating œstrogen, a lower one at which the secretion of lactogenic hormones is evoked and a higher one above which milk secretion is inhibited.

It may therefore be that relatively low levels of circulating œstrogen can excite the same centres in the hypothalamus as

*A full paper on this work has now appeared (Cowie, Folley, Malpress and Richardson, 1952, *J. Endocrinol.*, 8, 64)

those believed to respond to the suckling stimulus and with the same end result, namely that prolactin secretion by the anterior pituitary is evoked, possibly by a humoral excitant travelling along the hypophyseal portal vessels. Higher œstrogen levels would appear to inhibit, rather than excite the postulated centres; whether or not the antagonistic action of progesterone is also exerted at this point it is impossible to say. We thus have evidence for two alternative mechanisms which may govern prolactin secretion, each of which may function physiologically in different circumstances.

We are indebted to Dr R. W. Bates of Messrs E. R. Squibb & Sons for the purified prolactin used in the unpublished experiments mentioned in this paper

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DISCUSSION

NELSON: In your *in vitro* experiments on the mammary gland, where oxygen uptake was increased, did you add œstrogen as well as prolactin?

FOLLEY We haven't had time to do that yet, at the moment we are starting studies on the addition of prolactin and corticoids.

NELSON I was thinking of the possibility of inhibiting effects.

SONENBERG Have you investigated the effects of other proteins in the bathing fluid?

FOLLEY We have tried inert proteins—casein, serum albumin.

RAWSON Were these experiments on the rabbit mammary gland?

FOLLEY. No, on the rat mammary gland We have found similar respiration phenomena in mice, rabbits and guinea pigs. Ruminant udder tissue will utilize acetate as sole substrate and non-ruminant mammary tissue will not. The reverse is true of glucose as substrate. Mammary tissue from both types of animal will utilize acetate in the presence of glucose

CROOKE: We have found that lactogenic hormone may be present in male urine in similar concentrations to that found in female urine.

FOLLEY This brings up the question of the rôle of prolactin in the male. Have you anything to say about that, Dr. Nelson?

NELSON As I said earlier, our prolactin preparations apparently were contaminated with gonadotrophin. To the best of my knowledge the question you have asked remains to be answered.

JACOBSOHN. With regard to the action of œstrogen in promoting milk secretion, you think that the œstrogen might act on the hypothalamus?

FOLLEY: It is possible.

JACOBSOHN: My studies on the mammary glands of rabbits suggest that œstrogen can act directly on the anterior pituitary gland. In 1949 (*Acta. physiol. scand*, 19, 10) I observed that œstradiol benzoate given after a complete transection of the hypophyseal stalk produced peculiar changes in the mammary glands The gland appeared widely distended with a milk-like secretion, although there was little or no proliferation of the parenchyma This study was continued on 14 completely and 11 incompletely hypophysectomized rabbits and on

About one month after hypophysectomy one mammary gland was removed and the injections with œstradiol benzoate started. Mammary glands were then examined at different intervals after the beginning of the injections from about a week to three months In all the incom-

continuous involution. In only three out of the 14 hypophysectomized rabbits did I find ducts slightly distended with a colourless secretion. Thus, in rabbits with the hypophysis completely separated from the brain there is a secretory response to œstrogen which does not occur after hypophysectomy. This indicates that the pituitary gland, even after separation from the brain, mediates a stimulation of the mammary gland.

FOLLEY: Our *in vitro* experiments have shown that quite soon after weaning there are reverse changes in the respiration—the Q_{O_2} goes down

JACOBSOHN: Probably the metabolic rate reflects changes in activity more closely than does the microscope. As far as I could see microscopically, in rabbits secretion continued for several days after weaning as well as after transection of the hypophyseal stalk during lactation (*Acta physiol. scand.*, 19, 10, 1949).

DESCLIN. The influence of suckling on the pituitary has been shown histologically in experiments in which castrated rats which were suckling their young were compared with castrated animals whose young had been removed The pituitaries of the first group contained no castration cells, while those of the second group did contain castration cells In animals in which the pituitary stalk has been transected lactation ceases, but the mammary gland does not regress as it does in hypophysectomized animals, even though the portal vessels are interrupted.

JACOBSOHN: Similar experiments on rabbits confirm that the changes occurring in the lactating mammary gland after stalk transection are

JACOBSOHN. Unpublished experiments on rabbits injected with oxytocin after stalk transection do not seem to support this assumption

DESCLIN: I am puzzled about Gomez's experiment in which he treated hypophysectomized rats with prolactin and oxytocin, and found that the young could be reared until weaning. We have tried to repeat these experiments in a few trials which seemed to confirm Gomez's results.

FOLLEY: These experiments have been confirmed.

DESCLIN: We have done too few experiments, and they have not been published. I wonder if anyone has tried to duplicate this work.

COWIE: We have tried replacement therapy in hypophysectomized rats and found no replacement with anterior pituitary extract alone. We hope to repeat these experiments with anterior pituitary extract plus pitocin.

FOLLEY: We have done one or two experiments with neurohypophysectomized animals.

COWIE: I removed the posterior pituitary from two lactating rats. They had to be given injections of pitocin two or three times a day to allow the pups to grow normally. Without injections of pitocin the pups could obtain no milk at all from the mothers. Pitocin is essential for the discharge of milk from the mammæ.

HARRIS: In experiments performed in collaboration with Mr. B. A. Cross in Cambridge, we have found that following electrical stimulation of the pituitary stalk in lactating rabbits, there is an ejection of milk from a cannulated teat duct. This response has the characters of one mediated humorally, that is, long latency, slow rise to peak and so on, and may be duplicated very closely by an intravenous injection of posterior pituitary extract. Conversely, lesions of the supraoptico-hypophyseal tract caused interference with the normal course of lactation, and replacement therapy with posterior pituitary extract was effective in these rabbits with lesions.

KENNEDY: It would be nice to know how long interference with lactation persists after stalk section, as acute damage in this region often causes effects from which there is recovery with time. We have found that lesions placed in the tuberal region of the hypothalamus during lactation in the rat are followed by the death of the litter from starvation in about 50 per cent of cases. As the rats subsequently refuse to mate we could not study a further pregnancy, but we found that in most cases normal lactation could be re-initiated after an interval of two or three weeks by applying a suckling stimulus in the manner of Selye and McKeown's experiments. The temporary interference with lactation was independent of diabetes insipidus, and I doubt if it was a specific effect.

HARRIS: In these experiments there was no interference with milk *secretion*, but only with the *discharge* of milk already present in the mammary glands. There can be very little doubt now that there is a direct relationship between the function of the posterior pituitary gland and the *discharge* of milk, or milk-ejection, from the mammary gland.

KENNEDY: Was this correlated with signs of diabetes insipidus?

HARRIS: Rabbits do not appear to develop permanent diabetes insipidus. That was our finding, and we have been unable to discover any account in the literature of such a condition in rabbits. However, the interference seen in the milk-ejection process showed a close correlation with the site of the lesion in the supraoptico-hypophyseal tract, as seen histologically.

INTERACTION BETWEEN PROLACTIN AND PROGESTERONE IN THE REGULATION OF THE SECRETORY PROCESSES IN THE MAMMARY GLAND

L. DESCLIN

It is now generally accepted that the lactogenic hormone prolactin plays an important part in the development of the mammary gland in normal animals. This action is mainly an indirect one and results from the so-called luteotrophic activity of prolactin. Besides these luteotrophic properties, prolactin is still considered to have the ability to initiate the secretory activity of the gland characteristic of lactation.

If these two properties of prolactin are involved in the control of mammary activity under physiological conditions, the problem of their interrelations remains very obscure. In fact, every explanation of the initiation of lactation must take into account the now well established fact of the luteotrophic activity of prolactin.

In the last few years, we have had the opportunity to make certain observations which might be of interest for the understanding of this problem. Adult cyclic female rats were injected with a commercial preparation of prolactin containing 12 i.u. per mg. They received a daily dose of 3 mg. of this preparation injected subcutaneously. The fifth day after initiation of the treatment, a thread was introduced into both uterine walls. The animals were killed after ten injections.

Other adult female rats were spayed and injected exactly in the same way with prolactin. The injections were started on the day of operation. The uterine horns were traumatized equally on the fifth day as in the previous group Sacrifice after ten days.

In the first group, the uterine horns showed voluminous deciduomatous formation in nine out of ten animals. This result shows a very definite luteotrophic activity of the extract injected. In the second group of animals, the genital tract was atrophic, without any deciduomatous reaction.

The comparative study of the histological picture of the mammary gland in these two groups of animals was very instructive. In the first group, we found regularly a very intensive lobulo-alveolar proliferation and a picture which might be compared with the state of development attained during the second third of pregnancy. As a rule, the structure of the gland is very compact. The lumina of the alveoli are not conspicuous. The secretory activity of the epithelium is very poor and the secretory products accumulate in the cells and are not released into the lumina, which have a collapsed appearance. In all these animals, the histological study of the vagina reveals an intensive mucification which may be compared with the vaginal mucification of pregnancy.

In the spayed animals injected from the day of spaying with the same doses of prolactin, the picture was entirely different. The mammary glands after ten days were not so intensively developed as in the normal animal similarly treated, but were certainly not regressing as they do after castration. The most striking difference resulted from the distension of the alveoli whose lumina were filled with secretion products, which were also found in the distended ducts.

This difference in the type of reaction of the mammary gland to the same dose of prolactin in the normal and in the spayed animal seems to point to the presence in the ovary of inhibitory factors. That the stimulated corpus luteum might be responsible for this inhibition of the secretory activity of prolactin is obvious.

We might find a confirmation of this idea in the study of normal rats injected with prolactin. Among these, a certain percentage of animals do not show the luteotrophic activity of prolactin. Their uterine wall has not reacted to injury by deciduomatous formation and the vaginal mucosa is not

mucified; sometimes it is even keratinized and in full œstrus. Study of the mammary glands of these animals, where the corpora lutea have not been stimulated, is very interesting. They are equally well developed but also actively secreting, with numerous distended alveoli and ducts filled up with secretory products, contrasting sharply with the intensively proliferated, poorly secreting glands of the animals with active corpora lutea. We may conclude that prolactin is able to exert its secretory activity in the mammary gland only if it does not exert its luteotrophic action.

An inhibitory action of the corpus luteum on lactation has been often uggested. In fact it has been usually interpreted as an inhibition of the secretory activity of the anterior lobe, an inhibition of the release of prolactin through the pituitary. This interpretation seems not to be entirely satisfactory to explain our results.

The same contrast in the type of reaction in the mammary gland may also be observed in experiments where instead of using prolactin, the animals are treated with œstrogen. It is well known that œstrogen administered to normal rats has also a luteotrophic action on the ovary. A state of pseudo-pregnancy may result from this treatment.

In a series of rats, normal or castrated, implanted with stilbœstrol tablets and sacrificed after ten days, we could find in the mammary gland the same changes as in the prolactin experiments: an intensive lobulo-alveolar development without secretion in normal animals possessing active corpora lutea; on the other hand, an active secretory activity with distension of the alveoli of the spayed animals, or of those whose ovaries did not contain active corpora lutea and were in an œstral condition. Here again, we have another example of the inhibitory action of the corpus luteum on the secretory processes in the mammary gland.

A similar inhibition has been found by Selye with combined injections of progesterone and œstrone in castrated rats. Administering huge amounts of progesterone alone (15 mg. daily) to castrated female rats he succeeded also in obtaining

a very intensive lobulo-alveolar development of the mammary gland. He insists on the compact appearance of the tissue and an absence of secretion.

In view of these results it might be that in our experiments with prolactin, the progesterone secreted by the stimulated corpora lutea holds in check the secretory activity of prolactin.

An alternative explanation could be that, through a sort of partition of the available hormone, prolactin used at the level of the corpus luteum to stimulate progesterone secretion would not be present in sufficient amounts to induce lactation in the mammary gland at the same time. This would suppose a kind of competition between the receptors for the use of prolactin.

New results obtained by us in the last months might be of interest in connection with these theoretical views. We repeated our experiments with prolactin injections in normal and spayed adult rats with the same result as previously described. Besides these control experiments, in other castrated adult female rats we injected simultaneously, from the day of castration on, a daily dose of prolactin 30 i.u. plus progesterone 10 mg. The animals were killed after 10 days. Both hormones were administered subcutaneously but separately at different sites of injection.

The mammary glands of these rats, after ten days of castration, were very well developed and showed many lobules with numerous alveoli. In fact they were much better developed than those of the spayed animals treated with the same dose of prolactin alone for the same time. But the type of reaction was the same as in those. Secretory processes were very active. Alveoli and ducts were excessively distended and filled up with secretory products. In fact, far from having restrained the secretion of the gland, the administration of progesterone enhanced the secretory processes in the breast.

We have tried to repeat the same experiments in hypophysectomized rats, normal or spayed at the time of hypophysectomy. These experiments are rendered very difficult in view of the very rapid involution of the mammary gland

which follows the removal of the pituitary body even when rather pure lactogenic hormone is injected. To avoid as far as possible this inconvenience, we performed hypophysectomy and castration on the fifth day of treatment.

In normal animals injected daily with 30 i.u. of prolactin, hypophysectomized the fifth day after the treatment has been started and killed on the ninth day, the alveolar development of the mammary gland is very poor and cannot be compared with the intense growth obtained in normal animals under similar conditions of treatment. The same is true for hypophysectomized castrated animals. In the series of rats treated with prolactin, hypophysectomized and spayed on the fifth day and then injected with 10 mg. progesterone daily, the development is much better. Many alveoli are present and secretion is conspicuous. Here again, it may be concluded that progesterone has not inhibited the secretory activity of prolactin but rather enhanced this action.

If we try to summarize the results of our investigations we might say that prolactin administered to normal or castrated rats induces in the mammary gland very different reactions. The secretory processes are very marked in the absence of the ovaries or if the corpora lutea have not been stimulated to secrete progesterone.

This inhibition of secretion might result from the action of progesterone directly on the mammary gland or indirectly by way of the pituitary. The administration of prolactin and progesterone together to normal or hypophysectomized castrated rats does not reveal this inhibitory effect of progesterone on the secretion of the breast. On the contrary, the secretory processes seem rather enhanced under the influence of the progesterone treatment.

The alternative explanation that partition of prolactin between mammary gland and corpus luteum might be the reason for the difference in the reaction of the breast in normal and castrated animals submitted to the same treatment with lactogenic hormone, though not at all proven by our observations, remains in keeping with the facts observed.

Acknowledgement

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DISCUSSION

FOLLEY: In the beginning of your paper you said that prolactin promotes not only secretion but growth of the mammary gland?

DESCLIN. Yes, it acts via the ovaries to produce a marvellous growth

not only was secretion induced, but also some hyperplasia of the mammary gland.

DESCLIN. My impression was that even in the castrated animal

FOLLEY: So you now favour the concept of the target organs competing for the utilization of the hormone.

SMELSER: In regard to the possibility of competition, if you give two or three times as much prolactin, do you get results in rats with intact ovaries?

DESCLIN: We haven't done that so far. Experiments of this kind are underway.

NELSON If prolactin is not the pituitary factor involved in the growth of the mammary gland, then which pituitary factor is involved? It is very difficult to produce mammary growth in hypophysectomized animals with œstrogen and progesterone.

DESCLIN: I agree. In hypophysectomized animals we get growth

prolactin, at the end of this growth period.

JACOBSOHN: In hypophysectomized rabbits I could not find mammary gland growth after administration of œstradiol benzoate and progesterone.

FOLLEY: Fredrikson* claimed that œstrogen produced mammary growth in completely hypophysectomized rabbits.

JACOBSOHN: The evidence presented by Fredrikson does not seem quite convincing. To mention one thing, Fredrikson examined sections taken from a "medial sector" of the mammary gland and did not study whole mount preparations. My experiments are not quite comparable to Fredrikson's. He started injections a few days after hypophysectomy, whereas I waited about a month until an atrophy was established.

WILLIAMS. You mean that after hypophysectomy one must allow the mammary gland to regress?

JACOBSOHN. Yes, if you want to get a defined condition at the start of the experiments. The degree of mammary gland [illegible]

according to whether it is atrophic or not

JACOBSOHN: Fredrikson claims that the reaction of the mammary [illegible]

begun as late as five months after operation

*Fredrikson, H. (1939) *Acta obstet gynæc. scand*, 19, Suppl 1.

HYPOPHYSEAL AND OVARIAN HORMONES IN THE REGULATION OF THE MAMMARY GLANDS

WARREN O. NELSON

In the consideration of this subject the material to be presented will fall into two related but distinct categories—the factors that are involved in growth of the mammary glands and those that are concerned with the secretion of milk. Recent reviews of the subject written by Folley and Malpress (1948) and Mayer and Klein (1948) provide painstaking consideration of the abundant literature on the mammary gland that has appeared since the stimulus provided by Stricker and Greuter's (1929) demonstration of the relationship of the hypophysis to lactation.

Throughout this presentation constant reference will be made to a number of terms which should be defined at the outset. The terms FSH and ICSH refer, respectively, to the follicle stimulating and interstitial cell stimulating or luteinizing hormones. The term prolactin will be used in a generic fashion, but frequent reference will be made to its *three* activities related to mammary gland function. (1) Luteotrophic activity, i.e., its effect in promoting the secretion of progesterone. (2) Mammotrophic activity, i.e., its effect in promoting growth of the mammary glands. (3) Lactogenic activity, i.e., its effect on lactation. It is now apparent that none of the many names that have been proposed for this [illegible] ct of [illegible] able [illegible] the hypophysis in mammary growth and lactation. These various points of view were frequently the result of variations encountered in different species and have been resolved by

further observations. However, a number of basically important points have remained undecided. Among these has been the relationship between the ovarian hormones, œstrogen and progesterone, and the hypophysis in growth of the mammary glands. General agreement has been reached, in most quarters, that in the presence of the hypophysis the ovarian hormones will promote growth of the glands. However, major differences of opinion on the mechanism by which the hypophysis is related to the growth process have remained. Thus Turner and his colleagues (Lewis and Turner, 1939, Mixner and Turner, 1943) reported the existence of two separate hormones of the hypophysis which were considered to be responsible for the growth of the ducts and alveoli respectively. These hormones, called "mammogens," were regarded as distinct from all known hypophyseal factors and were said to be produced as a result of the stimulation of the hypophysis by œstrogen and progesterone. This concept relegated the ovarian hormones to the rôle of pituitary stimulants and did not accord them a part in the direct stimulation of the mammary tissues.

Although a number of weaknesses in this theory were pointed out by various workers, the most significant objection was provided by Lyons (1951) in a report to the International Conference on the Physiology of Lactation at Strasbourg in August, 1950, and additional evidence was presented at the same conference by Nelson (1951). Both of these studies, in which the rat was the experimental subject, emphasized the triple activity of prolactin, and it was shown that in the presence of adequate amounts of œstrogen and progesterone the only hypophyseal hormone which was required to produce definite lobule-alveolar development of the mammary glands in hypophysectomized rats was prolactin. Even better growth was obtained when ACTH and somatotrophin (growth hormone) were provided, but these hormones are not effective in the absence of prolactin and their activity may be regarded as due to improvement in the physiological state of the hypophysectomized animal.

Lyons employed various types of animal preparations including normal, oöphorectomized, hypophysectomized, oöphorectomized-hypophysectomized and oöphorectomized-hypophysectomized-adrenalectomized subjects in his studies and adapted the experimental procedures to the particular type of preparation. Of special significance were his experiments in hypophysectomized and oöphorectomized-hypophysectomized animals. In the former case he repeated the observation that combinations of œstrogen and progesterone would not promote lobule-alveolar development. The same failure occurred in animals which received FSH and ICSH; the ovaries of these animals showed definite stimulation and produced œstrogenic hormone, but not progesterone. When prolactin was given in addition to œstrogen lobule-alveolar growth occurred. It should be noted that in this experiment it was unnecessary to provide progesterone since prolactin through its luteotrophic effect on the ovaries stimulated the secretion of endogenous progesterone. In other hypophysectomized animals Lyons injected FSH, ICSH and prolactin. This combination of hormones stimulated the ovaries to produce œstrogen through the action of FSH and ICSH and progesterone through the luteotrophic action of prolactin. As a result of its mammotrophic activity the latter also provided the additional stimulus required for lobule-alveolar growth.

In further experiments on oophorectomized-hypophysectomized animals Lyons showed that the combination of œstrogen, progesterone and prolactin, or even better the prolactin-ACTH-somatotrophin complex, would induce excellent development of the mammary glands. Œstrogen with or without progesterone, prolactin, or the prolactin-ACTH-somatotrophin complex given separately failed to stimulate lobule-alveolar growth. These experiments provide very strong evidence against the "mammogen" theory which holds that the action of œstrogen and progesterone in mammary growth is an indirect one through the hypophysis in stimulating the secretion of the specific mammogenic hormones. Furthermore, they indicate that a recognized hormone of the pituitary,

prolactin, fulfills the requirements of a mammary growth stimulating hormone and that it is unnecessary to postulate the existence of distinct mammogenic hormones.

Lyons was able also to show that the rat placenta contains a substance that appears to be identical with prolactin in producing luteotrophic and mammotrophic effects. The administration of rat placentæ (from rats pregnant at least 12 days) would substitute for prolactin in the various experiments on mammary growth, and this finding was taken to indicate that in normal pregnancy the placenta plays an important rôle in the secretion of progesterone by the corpora lutea and in the stimulation of mammary growth.

In the experiments reported at the same time by Nelson essentially similar results were obtained. It was shown that animals made pseudopregnant by the administration of œstrogen possessed large, highly active corpora lutea and well developed mammary glands after 8 to 20 days of treatment. These changes were shown to be the result of release of prolactin under the stimulus provided to the pituitary by œstrogen. Through its luteotrophic action prolactin stimulated the secretion of progesterone, œstrogen was of course provided by the treatment procedure, and through its mammotrophic activity prolactin supplied the additional stimulus necessary for continued mammary development. That this sequence of events and combination of factors afforded the most reasonable explanation for lobule-alveolar growth comparable to that seen in late pregnancy wa shown by subsequent experiments.

(1) Animals were injected with œstrogen as above, but hypophysectomized 8 to 14 days after the initiation of treatment. In such cases the corpora lutea were not maintained and the mammary glands underwent involution.

(2) If hypophysectomy of the œstrogen treated pseudopregnant rat was followed by the daily administration of a good preparation of prolactin (75 to 150 i.u.) which also contained small amounts of ACTH and somatotrophin the corpora lutea were maintained and mammary development was continued.

(3) In animals hypophysectomized and oöphorectomized on the 8th day after the initiation of pseudopregnancy by œstrogen and subsequently treated with the same prolactin preparation, the mammary glands not only failed to continue growth but actually underwent involution. This experiment emphasizes the necessity of œstrogen and progesterone acting with prolactin to promote continued development of the mammary glands.

(4) Animals were hypophysectomized on the 8th day after initiation of pseudopregnancy and œstrogen treatment was *withdrawn at the same time; injections of chorionic* gonadotrophin and/or pregnant mare serum were begun immediately. Pseudopregnancy was terminated, but mammary gland involution occurred due to failure of progesterone production in the corpora lutea. However, if in addition to chorionic gonadotrophin and pregnant mare serum the animals received prolactin, the secretion of progesterone was maintained and mammary development progressed.

In subsequent studies, reported here for the first time, it has been possible to confirm several of the experiments described by Lyons. These include his observations on the type of mammary development which is seen in oophorectomized animals injected with œstrogen and progesterone and the failure of such treatment to induce lobule-alveolar growth in oophorectomized-hypophysectomized animals unless prolactin also is administered. Additional confirmation has been found for Lyons' observation that prolactin preparations which contain ACTH and somatotrophin are better mammotrophic agents than those which are deficient in these hormones. As noted earlier these hormones probably have no mammotrophic activity *per se*, but rather provide for restoration of the hypophysectomized animal's physiological state to some semblance of normal.

In summary it would appear that three factors have been shown to be directly involved in lobule-alveolar growth in the rat mammary gland. *These are œstrogen, progesterone and* prolactin, the last serving a dual rôle in stimulating the

secretion of progesterone by the corpus luteum and in some manner having a direct growth-promoting action on the mammary glands. Whether this latter activity is exerted by direct stimulation of the mammary parenchyma or through an influence on metabolic activities which are of fundamental importance in mammary development remains to be determined. However, it should be noted that evidence in favour of the former has been presented by Lyons (1942) who injected prolactin intraductally in rabbits that had been previously treated with œstrogen and progesterone. Localized hyperplastic changes including mitoses were observed in the sectors of the gland associated with the injected ducts.

Probably less controversy has existed over the hormones *which initiate lactation than those concerned with growth of* the mammary glands. However, there has been considerable difference of opinion about the interrelation of those factors which stimulate and those which inhibit lactation and about the factors which are involved in the maintenance of lactation.

Initiation of lactation in the intact animal would seem to depend upon the presence of prolactin and ACTH (Nelson and Gaunt, 1937) and according to Folley and Young (1940) and Bergman and Turner (1940) the augmentation and maintenance of established lactation depends upon a galaxy of hormonal factors. These two groups of workers differ in their views regarding the details of this complex problem, but in general it may be said that two distinct mechanisms are supposed to be involved in the lactation process. These are (1) lactogenesis, which is defined as the initiation of lactation in a well developed but non-lactating gland, and (2) galactopoiesis, which is defined as the maintenance or augmentation of established lactation. The distinction between these two processes and the possible factors involved in galactopoiesis have been discussed extensively in the recent review by Folley and Malpress (1948).

These same reviewers have discussed and criticized various theories that have been proposed to account for the hormonal factors involved in the initiation of lactation. One of these

was evolved from the earlier theories of Halban (1905), Frankl (1923), Fellner (1931) and Nelson (1934, 1937) to apply to lactation in the guinea pig. In brief it supposed that lactation is held in abeyance so long as the levels of œstrogen remain high, as in pregnancy, and that lactation occurs under the stimulus of prolactin when those levels are reduced at parturition. The mechanism whereby œstrogen inhibits lactation was regarded as involving the suppression of release of prolactin as well as a direct inhibition of the action of prolactin on the mammary glands. More recent experiments, some of which have been reported (Nelson, 1951), indicated that œstrogen exerts its most potent inhibitory effect in the guinea pig by directly opposing the lactation-inducing influences of prolactin.

These experiments were done on either normal or hypophysectomized female guinea pigs which had recently delivered litters, or on normal or hypophysectomized young adult male and spayed female guinea pigs which had received œstradiol for about 50 days. In either case hypophysectomies were done after parturition or after preliminary treatment with œstradiol had been completed.

Lactation occurs at the time of parturition, of course, and is initiated within three days following the cessation of œstrogen treatment in either males or females. If the hypophysis is removed lactation fails to occur or, if it has been initiated prior to hypophysectomy, it ceases soon after that operation. When prolactin and ACTH were injected in these animals lactation was initiated or maintained.

However, when œstradiol in daily doses of 0·1 mg. was injected in these hypophysectomized animals under prolactin-ACTH treatment lactation either failed to occur or was terminated during the period of œstradiol treatment. These experiments and similar ones in intact animals indicated that œstrogen exerts an important lactation-inhibiting action by direct action on the mammary glands. This action has been visualized as one in which the lactation inducing effect of prolactin may be interfered with by the maintenance of an

active stimulus for growth by œstrogen. However, it is also possible that the inhibitory effects may be due to an interference with some metabolic activity of the alveolar cells essential to the lactation process.

If this concept of two opposing mechanisms, one concerned with the promotion of growth and the other concerned primarily with the initiation of a secretory process, is in any measure representative of the facts it is reasonable to suppose that it should be possible, by suitable adjustment of the two mechanisms, to throw the controlling influence in either direction. Thus it was noted that when the daily dose of œstradiol was reduced from 0·05 mg., the amount used to promote growth of the glands in intact animals, to 0·01 mg. daily, a scanty lactation occurred on the fourth day in 3 of 14 animals. In the remaining 11 animals lactation had not occurred on the sixth or seventh day. When injections of prolactin (250 to 400 i u daily) were initiated, lactation did occur in all these animals on the second or third day of treatment. These results were regarded as indicating that œstrogen can be present at a level inhibitory to the spontaneous lactation which follows œstrogen withdrawal, but not inhibitory to extra quantities of prolactin. It may be that such a level of œstrogen maintains minimal growth stimuli, or minimal interference with metabolic processes important to lactation, and so interferes with the animal's own prolactin. However, that level of œstrogen is unable to inhibit the effect of large amounts of added prolactin.

These experiments and others reported previously (Nelson, 1951) are regarded as demonstrating the existence of a sort of "push-pull" mechanism in the control of lactation in the guinea pig and to indicate that peripheral interference with the action of prolactin by œstrogen is the principal mechanism whereby the latter inhibits lactation.

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DISCUSSION

FOLLEY In those experiments on pseudopregnant rats which you hypophysectomized, you didn't include any groups of adrenalectomized animals? I ask that because I believe you were using a prolactin preparation provided by Dr. Bates, and we've had what I imagine is almost certainly some of the same prolactin, which, Dr. Bates told us, contained about 20 per cent ACTH It seems to me that if the adrenal cortex has any rôle in mammary growth, you might throw light on that by taking out the adrenals as well as the hypophysis.

NELSON: Our recent experiments were made with that particular preparation, but the basic ones were done eight or nine years ago in Detroit, some time I am sure before Dr. Bates made that particular preparation. I know that in at least one or two of those preparations there was a very low ACTH content, as far as we could tell by the assay procedure we used at that time, namely the repair of the adrenals of the hypophysectomized rat Your suggestion would be most valid for this particular preparation which has been supplied by Dr. Bates recently. It does have appreciable amounts of ACTH, and that was important to the results we obtained in the studies on lactation. Many of the latter were done with that preparation, and as you know we needed ACTH to obtain lactation in the hypophysectomized guinea pig.

COURRIER: I was wondering if the relation between the lactogenic hormone and œstrogen might not be referred to a very simple process. According to A. Prenant we know that cells which are dividing do not

secrete. Perhaps the œstrogen, which stimulates the growth of the alveolar cells, may thus inhibit the induction of lactation.

NELSON: That is the simplest explanation, namely, that while the gland is under the influence of an active growth promotor it is not likely to be a secretory gland. However, I suspect that probably it is more complex than that. For example, it may be that œstrogen interferes by some competitive mechanism within the alveolar cell with the action of prolactin in the induction of lactation. That is the reason I am very eager to learn what Dr. Folley discovers when he adds œstrogen together with prolactin in his studies of oxygen uptake in the mammary gland.

FOLLEY. I think there are further difficulties with that suggestion, because in our experiments on artificial udder growth in goats it is [illegible]

SPENCE. May I ask about the action of œstrogen during pregnancy in the suppression of prolactin? I think you said that during pregnancy the œstrogens suppressed the prolactin secretion, and after delivery the œstrogens fell and then prolactin was liberated. I understood that the œstrogens during pregnancy were conjugated and inactive, [illegible]

prolactin secretion.

NELSON. Years ago when I first did experiments on lactation in the [illegible]

pituitary may be a factor, the more important mechanism is probably the inhibition of lactation at the level of the mammary gland. As far as inhibition of the pituitary by œstrogen is concerned, one cannot generalize. In the rat it can be shown that œstrogen stimulates the release of ACTH, and it certainly stimulates the release of luteotrophin [illegible]

[illegible] Nelson, I think that most of your experiments have been [illegible]

[illegible] produces initiation of secretory processes, whereas in the normal

animal it only induces growth. I think the situation is probably different in the rat and in the guinea pig. We know that in the guinea pig œstrogen alone can give complete development of the mammary gland. Perhaps your view might be corrected as far as the rat is concerned.

Nelson. I don't believe this hypothesis can be applied to the rat without modification. Lactation in the rat, in my experience, is a much more complicated problem than it is in the guinea pig.

Richardson: I should like to ask Dr. Nelson if it is at all feasible to inject œstrogen into the ducts of the guinea pig's mammary gland. If it could be confined to a localized part of the duct system, as Lyons did with prolactin, one might expect to see inhibition in that part of the gland and not elsewhere.

Nelson: I haven't investigated that possibility. I don't think it would be as easy as in the rabbit, which has a more diffuse type of mammary gland than the guinea pig, which has a very compact gland.

Williams: How do your guinea pigs survive hypophysectomy when compared with rats? In my experience they're rather difficult to handle.

Nelson: Yes I agree with you. They are much more difficult than the rat, but by no means as difficult as the rabbit to nurse through the post-hypophysectomy period.

BOOK II

HORMONAL INFLUENCES IN WATER METABOLISM

CHAIRMAN'S OPENING REMARKS

S. ZUCKERMAN

At the outset I have to thank the Trustees of the Ciba Foundation, through Dr. Wolstenholme, for their very great kindness in having organized this meeting, and to extend a welcome from the domestic members of the conference to those of you who have come from abroad, some of you from as far away as the other side of the United States

The fact that I have been chosen as chairman does not mean that I have any great knowledge of the subject we are to discuss, and I must warn you in advance that I have not made myself up-to-date in your own special fields of work. My hope is that I shall become so as a result of our meeting. What I can say with confidence in introduction is that this conference represents a mark of great progress. Fifteen or twenty years ago there was practically no reference to steroid hormones in studies of body water; the great diversity of the programme in front of us is an indication of the advances that have recently been made. My personal interest in these studies goes back before the days when the steroid molecule was chemically defined, for I was even then interested in a rather peculiar phenomenon which has continued to divert me ever since—namely, the swelling and subsidence of the hindquarters of the baboon in phase with the menstrual cycle—a phenomenon which we now recognize as an effect of stimulation by the ovarian steroid hormones. I realized that it was a manifestation of a special type of œdema, but in so far as it appeared to have no connection with the œdema which physiologists were then studying, I failed to persuade several learned friends that it was a problem worthy of study. And unfortunately I was too little of a physiologist to carry its analysis very far myself.

To-day we see the problem of water metabolism as involving not only the gonadal and adrenal steroids, with which this conference is mainly concerned, but almost all the endocrine organs, and particularly the two parts of the pituitary. And as we all realize, water metabolism is also conditioned by a number of other factors, known and unknown.

Looking at the whole field as a non-specialist, I feel it may be useful not to regard the shift of water and electrolytes as something which should be viewed in complete isolation. It represents only an aspect of general changes that are related to the activity and growth of tissues. While it is true that in order to advance our subject we have to isolate the particular phenomenon in which we are most interested, it is unwise, as experience has repeatedly shown, to persist in the assumption, if I may take a particular illustration, that there is one lot of corticoids in the adrenal concerned only with water metabolism and electrolyte balance, and another, the glucocorticoids, which are concerned solely with other aspects of metabolism. Experience indicates that sooner or later the two fit into the same picture.

The title of our conference is "The effect of steroids on local and general water distribution." It comprehends a number of problems. One may assume first, that the reference to local water distribution implies that different tissues may be specialized in different ways with respect to the retention of water. Thus, sexual-skin swelling is a specific kind of water retention controlled by gonadal as opposed to adrenal steroids. Local distribution may therefore imply the specific adaptation of specific tissues. Furthermore, the water which is retained in tissues as a result of the action of one steroid is not necessarily retained there by another water-retaining steroid, so that we may suppose that very slight changes in molecular configuration have profound influences upon both local and general water distribution and electrolyte balance. Local changes also imply general consequences in water metabolism and *vice versa*. The behaviour of the kidney

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THE BIOLOGICAL EXCRETION OF SODIUM AND ITS RELATION TO CELLULAR WATER

E. J. CONWAY

THE active excretion of NaCl, or more specifically of Na ions, across cell membranes has been a necessary concept for certain groups of cells which, in higher organisms, regulate the electrolyte composition of the internal medium. Thus, one may cite the active excretion of chloride by the gills of fishes as shown by Keys (1931) and by Krogh (1936), the active reabsorption of Na ions across the distal tubule of the renal nephron as shown by the Richards school (1937), or the active uptake of Na ions by the skin of the frog, studied by Krogh (1938) and in recent years by Ussing (1949).

But it is now evident that those cells not directly concerned with the maintenance of the electrolyte composition of the internal medium have also this power, though not exercised in so pronounced a fashion. Thus it was shown by Conway and Hingerty (1946) that the skeletal muscle of the rat into which sodium had extensively entered during about a month's feeding on a K-free diet, was able to excrete the excess sodium when the animals were restored to a high K diet. The plasma potassium was restored to a normal level within 24 hours and subsequently to very high figures, but it required about three days for the excess muscle sodium to be half excreted, and normal levels were not reached for eleven or twelve days.

Such a slow excretion of sodium may be compared with the relatively very rapid mixing of such muscle sodium with labelled Na as shown by Heppel (1940) in Fenn's laboratory. Reasons may be advanced to account for such a rapid rate of isotope mixing combined with a very slow rate of active excretion, but it is not proposed to discuss these in the present communication. It appeared that skeletal muscle, constituting for the normal animal much the greater fraction of the

tubule may be one aspect of a change; the general retention of water in tissues another; and retention in certain specified tissues a third phenomenon, due to the action of a single specific substance.

The existence of these different aspects of a single wide problem is indicated quite clearly by the titles of the papers we have before us and which we shall be hearing these next two days. In general they seem to fall into two main groups, the first of which, illustrated by Professor Conway's paper on the biological excretion of sodium ions and its relation to cell water, concerns the underlying mechanisms of water shift, and the second the specific effects of different hormones and endocrine organs on water balance.

The wide range of our programme indicates the great progress that has been made in this field of knowledge in recent years, and I am quite sure that the next two days will provide a valuable opportunity for the cross-fertilization of ideas, all bearing upon our central problem, that of water metabolism.

While such ideas would lead to the conclusion that the active extrusion of Na ions was likely to be a general biological endowment, we were none the less surprised to find it in high degree in the yeast cell (*Saccharomyces cerevisiæ*) and the present communication is chiefly concerned with the presentation of the facts about such active sodium extrusion where it can be studied with ease on a single and very robust cell species. The preliminary study of such active extrusion (Conway and Ryan, 1950) appears to advance the solution of the problem of its operation a step further.

The Preparation of Yeast Containing a High Content of Na Ions (Referred to as NA-yeast)

In ordinary washed baker's yeast (*Saccharomyces cerevisiæ*) the Na content is very low—some few m. eqs./Kg., and may be vanishingly small.

If such yeast be suspended in N/10 NaCl solution containing labelled Na ions, there is no appreciable entrance of Na over many hours. If the suspending medium is an equal volume of 5 per cent glucose containing N/10 NaCl, very little Na likewise enters, compared with the corresponding K entrance in exchange for H ions when the NaCl is replaced by KCl. If, however, the suspending fluid be 5 per cent glucose with M/5 sodium citrate, which corresponds to an external Na ion concentration of 600 m. eqs./litre and the *p*H be buffered to above 5 0, a considerable amount of Na ions enter in exchange for H ions (Conway and Winder, 1950).

Such Na ions will be electrically balanced inside the cells by bicarbonate ions, also by the increased negative charges on the proteins, phosphate esters, etc.

In this way, by suspending it in 20 times its volume of 5 per cent glucose and M/5 sodium citrate for two hours and washing subsequently twice with 20 times its volume of tap water, the yeast is found to contain about 55 m. eqs. Na per Kg.

It may be possible by proceeding in this way to eliminate completely, or nearly so, the entire K content of the yeast and

soft tissue of the body, had the power to excrete sodium ions The same would appear to be true for conducting nerve as a consequence of the entrance of Na ions during excitation as developed by Hodgkin (1947) and by Hodgkin and Huxley (1947), and evidence for the active excretion of Na ions by mammalian erythrocytes has been advanced by different workers (reviews by Krogh, 1946, and by Ussing, 1949).

General Significance of the Power of the Cell to Excrete Sodium Ions

With regard, for example, to the power of skeletal muscle fibres to excrete sodium ions actively, it is to be noted that with a concomitant Cl permeability, and an entrance of Na^+ and Cl^- however slowly, provided the rate is significant with respect to the life of the fibres, there must occur an active excretion of Na ions, otherwise the fibres would necessarily swell indefinitely. It is true that this might be compensated by active water excretion, but as more and more NaCl entered the energy expended would be great, and a much more effective energy-saving process would consist in the reduction as far as practicable of the Na permeability, combined with the power of active excretion of entering Na ions. This corresponds to the actual endowment of the muscle fibres.

Similar considerations apply to all cells immersed in an early or late marine environment, or in the internal medium of the higher animals.

For the cell in general, complete impermeability to Na ions combined with a relatively easy passage of K ions is doubtless impossible, but a remarkably near approach has been made to it (Conway and Hingerty, 1948). For the animal cell with freely distensible cell membrane, the distinction between the permeabilities of the two ions may be referred to the greater size of the sodium ion, due to greater hydration, for there is a general but not a close relation between ion size and permeability. Whether this operates with respect to the concept of molecular pores or by relative displacements at surfaces is immaterial to the present issue.

There is an almost equivalent uptake of K ions (the K uptake usually exceeds somewhat the Na extrusion).

The process of Na extrusion here involves the expenditure of energy, since Na is being excreted into a concentration upwards of five times that remaining in the yeast cell. Also the K ions pass inwards to higher concentration levels.

The process of extrusion is somewhat faster when only N/10 KCl is present outside. It occurs also if the Na-yeast is suspended in tap water but only to about one-third the rate that in N/10 KCl or in N/10 KCl plus N/10 NaCl.

The excretion into tap water must be accompanied by anions from the yeast cell, which are likely to be succinate ions, but this has not been determined, and other anions may be involved.

The Unidirectional Nature of the Na-permeability during the Excretion

If the washed Na-yeast be suspended in a solution containing N/10 KCl and N/10 NaCl, the Na or K ions both being labelled, it is found that during the few hours' period when near 70 per cent of the sodium is excreted, almost no labelled Na enters the cells. Expressing the degree of mixing by the percentage ratio of the specific counts inside to outside, this amounts to only a few per cent. At the same time labelled K mixes very quickly with the whole yeast potassium. This is illustrated in Fig. 2, showing that in the first two minutes about 60 per cent ratio for the specific counts of K was found, but after some hours there was found only a few per cent ratio for the labelled Na

In other experiments the ratio for labelled K was even greater after two minutes, approaching and even exceeding 100 per cent, though such excess may be attributed to some degree of error in the procedure.

The actual counts registered for sodium throughout were very little above the background count. Thus the permeability is in effect unidirectional.

prepare a full Na yeast, just as we have prepared a full ammonia-yeast by another method (Conway and Breen, 1945). Meanwhile, for convenience, this yeast so prepared and containing when washed about 55 m. eqs. Na/Kg., will be referred to as a Na-yeast.

Demonstration of the Active Extrusion of Na from the Na-yeast

When such washed Na-yeast is immersed in N/10 NaCl (20 vols. per unit vol. yeast), practically no Na is extruded over many hours shaking at room temperature.

This may be contrasted with the effect of suspending in a similar volume of solution containing not only N/10 NaCl but N/10 KCl as well, under similar conditions (Fig. 1). It will be seen that the Na is now rapidly excreted from the yeast cells.

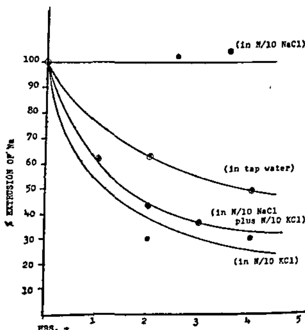


FIG. 1. Active extrusion of Na from Na-yeast in 20 times its volume of suspending fluid at room temperature.

The results of a preliminary set of experiments are given in Table I.

Table I

EFFECT OF INHIBITORS ON ACTIVE NA EXTRUSION

Na yeast washed and re-suspended in 20 times its volume of N/10 KCl with or without inhibitor (N/1000)

	Na content of yeast (m. eqs./Kg.)		
	Zero time	*After 2 hrs*	*Per cent fall of Na in yeast*
No inhibitor	44 7	19 3	57
Monoiodoacetic acid	44 1	18 0	59
Azide	44 8	29 5	34
Cyanide	44 1	33 0	25

It will be seen that both the azide and cyanide strongly inhibit the excretion, but monoiodoacetate under these conditions does not appear to inhibit. Such results, however, are subject to further confirmation and investigation.

The Nature of the Active Excretion of Sodium

From the evidence presented it appears that the excreted sodium does not traverse the cell wall or membrane as the free hydrated cation. It passes across the membrane therefore either as the non-hydrated ion or as a complex. If it passes as the non-hydrated ion, this should imply that, at least up to the membrane edge, it is likewise carried in bound form. But even if released at the membrane boundary, the fact that the diffusion velocity through the substance of the cell may be presumed to be very much greater than through the membrane, then for any degree of efficiency to be attained, it would seem necessary in turn to suppose that Na is carried into the molecular region of the membrane at least so far that the back diffusion into the cell is not relatively greater. The one possibility therefore merges into the other.

The Nature of the Carrier

When the magnitude of the sodium excretion is considered, upwards of 50 m. eqs./Kg., much of which is excreted in the

Such unidirectional passage of Na ions might be attributable—if we were dealing with the Na data alone—to a high potential gradient, but from the associated K data this is clearly untenable. It can then be attributed only to outward passage across the membrane occurring in some other form than the free hydrated sodium ion. In other words the Na ions appear to be carried in some way.

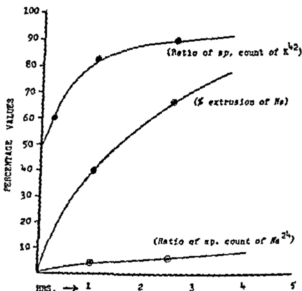


FIG. 2. Unidirectional passage of Na during suspension of Na-yeast in N/10 KCl and N/10 NaCl, Na or K ions being labelled, and percentage ratios calculated for labelled ions inside/outside cells.

The Effect of Some Respiratory Inhibitors on Active Na Extrusion. (Some Preliminary Observations)

The inhibitors used in these few preliminary observations were cyanide, azide and monoiodoacetic acid, each in N/1,000 strength. Part of the washed Na-yeast was suspended in 20 times the volume of N/10 KCl without inhibitor and similar amounts of the Na-yeast in 20 times their volume of N/10 KCl containing N/1,000 inhibitor.

A difficulty of interpretation would lie in the specificity, or some degree of specificity, required for the sodium ion binding In this connection it may, perhaps, be worth while recalling the determination of sodium and potassium ion concentrations by chemical procedures and the high degree of differentiation existing between insoluble complexes and the free ions of Na and K. In these complexes the Na and K components may be thought of as ionized, just as Na and Cl are ionized in solid sodium chloride.

Whatever the outcome of such interpretation may be, reference may be made to a recent paper by Steinbach (1950), who in a well planned series of experiments has advanced evidence for the existence of preferential binding of Na in the centrifugates from frog muscle homogenates.

The General Nature of the Hormonal Effect on Na Metabolism in Higher Animals

Here only the briefest treatment is possible It will be seen that with such a general cellular endowment as the active excretion of sodium, and essentially of the nature of a redox cycle or immediately dependent thereon, such hormones as those of the adrenal cortex can be considered as having only a controlling action by way of activating or inhibiting the enzymes involved, or, alternatively, by affecting the permeability of the cell membranes to Na and K ions.

With respect to the kidneys if the membranes of the distal tubule cells were rendered likewise more permeable to Na ions, then it may be assumed that Na would be conserved more readily with, again, the reverse effect after adrenalectomy or in Addison's disease. Applying the same considerations to K ions and the kidney, if the cells actively excreting K ions have their membranes more permeable to K ions, these may be assumed then to be more readily excreted and a low plasma K to result, and so on. However, a direct effect on the redox carrier systems may afford a better explanation.

first hour of the suspension in N/10 KCl, it may be assumed that the carrier is not excreted out with the sodium but operates in a cycle of carriage and release in the cell wall or membrane.

Pursuing the matter further from this stage, the carrier substance may be regarded as of one of two kinds:—

(*a*) The sodium is carried in a completely unionized state, sharing an electron with its carrier; the undissociated salt fraction of a polyphosphate may here be considered as an example.

(*b*) An electro-adsorption complex with a respiratory catalyst.

Either of these hypotheses has its advantages and disadvantages for the interpretation of the facts. Concerning the first possibility, recently Van Wazer and Campanella (1950) have brought strong evidence for the existence of phosphato-sodium complexes in solution, as well as for the relative binding capacity of certain polyphosphates for various metals. For the alkali metals the binding capacity goes as



$$Mg > Ca > Li > NH_4 > Na > K$$

Such polyphosphates have very high energy content and if the complex is broken enzymatically to release the Na ions a very high ratio of energy change to sodium excreted would appear necessary. Re-absorption of the phosphate would be required and also the regeneration of the energy rich polyphosphate. Likewise, the high degree of inhibition produced by N/1,000 cyanide, presumably upon a metallic respiratory enzyme or enzymes with high redox potentials, is not favourable for the interpretation through a polyphosphate cycle.

On the other hand the formation of a complex with a metallic respiratory enzyme has the advantage of immediately explaining the sensitivity to cyanide as well as affording a direct link between electrical energy and the performance of osmotic work. Thus a redox potential of such a system may be presumed to change in relation to the osmotic work performed in the stage of oxidation, as the potential of a flavine system alters with the H ion concentration.

six weeks on a potassium-free diet. As to the second question, we haven't any experimental data on relative functioning. They would be of much interest.

GINSBURG: In making the sodium yeast, can you increase the rate of sodium uptake by the yeast by using the polyphosphate complexes, which may enter the cell more quickly?

CONWAY: We haven't tried it. It is possible but I think it isn't likely, owing to the very high polarity of that phosphate complex. We did try to get in other phosphate compounds years ago, hexose phosphate esters and so on, in conjunction with sodium and potassium, but they didn't enter.

EICHELBERGER: I'd like to know, Professor Conway, if you have tried changing the pH's of your solutions or did you always work at the same pH?

CONWAY: In sodium entrance we have varied the pH extensively, but we haven't yet varied the external pH with the active extrusion. These are very recent experiments.

COLE The thing that strikes me about these experiments with yeast is that one seems to be able to do a lot more with the pH of the external medium than one can ever do with animal cells. With a pH of less than 2 one can still have a perfectly healthy yeast Presumably the balance between the external and internal pH and the external and internal concentration of sodium and potassium must show some very fundamental difference from the average animal cell. So although there must be some basic similarity, there must also be a fundamental point of difference there.

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DISCUSSION

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experiments* in particular, and also from some experiments I have done myself, seems to indicate the contrary, that there is a loss of sodium from tissues, muscle tissue particularly, that is *not* balanced by a loss of chloride. If the effect were simply due to a fall of chloride and sodium in the plasma, the chloride and sodium in the muscle should fall to a similar extent; but in fact the sodium seems to fall much more than the chloride does, which would indicate an active effect [illegible]

adrenal tends to balance up any changes that occur in the body, or whether there is some other factor involved, I don't know. [illegible]

the other hand, if the permeability is damped down and the mechanism still pumping away, you will have the reverse effect. That's not to say [illegible]

I wondered whether the content of water was normal throughout.

CONWAY: Offhand, I cannot give you the exact data for that, but the changes were not impressive.

SCHILD. There was one point I wasn't quite clear about. You had to have potassium outside in order to get your sodium "activated." Does that mean that the potassium actually went in in exchange for sodium? In that case it would not necessarily require free energy if it was only [illegible]

necessarily proves an active extrusion process, because cyanide alters the cell membrane constitution. [illegible]

to produce any serious effects on the membrane *per se*. Now concerning the condition of sodium in the cell, you raised the question that it may be localized in the cell?

* [illegible] F. Mellors, R. C. Mautz, F. R., and Mangun, G. H. [illegible] bbot, W. (1942). *J. biol. Chem.*, [illegible]

SCHILD: Yes. I am thinking in terms of an ion exchange.

CONWAY: All I can say is that if there are 50 milliequivalents of sodium in the yeast cell it is reasonable to suppose that they exist free just as in animal tissue cells. If they were bound it would indeed be a difficult matter to consider just how they were bound. It would require an ion exchange process with a very high degree of specificity for sodium. There's a lot of potassium inside the cell.

REGULATION OF INTRACELLULAR POTASSIUM AND RELATIONSHIP OF THIS REGULATION TO RENAL EXCRETION OF SODIUM AND POTASSIUM

J. A. LUETSCHER, Jr.

THIS introduction of the subject of cellular potassium will be somewhat oversimplified, since the clinical observer deals with only the most obvious factors in a complicated situation, using rather crude but laborious methods to obtain a minimum of basic information necessary for the treatment of the patient. The balance of intake against output and the analysis of tissues are so tedious that they provide only retrospective help. For the care of the patient, we must use rough estimates of intake and output, the level of potassium and of other electrolytes in the serum, and the manifestations of extreme variations in the ratio of extracellular to intracellular potassium concentrations on neuromuscular function as observed in voluntary muscle and in the heart.

In the normal man, a generous supply of potassium enters the body every day with the ordinary diet of natural foodstuffs. When this intake is reduced by refusal of food, by an artificial diet, or by serious disease of the gastro-intestinal tract, a gradual depletion of cellular potassium ensues. In most cases, deficiency of potassium is rapidly corrected when food intake returns to normal. Less commonly, excessive loss from the gastro-intestinal tract or from the kidneys is responsible for potassium depletion.

The potassium absorbed from the dietary intake enters the blood stream and becomes available to the extracellular fluid from which it may be taken up by the cells. The concentra- ... fluids is maintained at a ... active renal excretory ... cope with the ordinary

variations in potassium excretion and which can adapt to secrete very large excesses under unusual conditions. It is rare to see potassium intoxication except when renal excretory function is impaired by disease or by severe functional derangement. Less commonly, deficiency of potassium may occur by excessive renal excretion, usually in response to some stimulus which forces the kidneys to excrete potassium, rarely due to disease of the renal tubules with chronic acidosis. A rough estimate of the rôle of the kidneys in a given situation may be made by a comparison of the serum level with the rate of urinary excretion of potassium. If the kidneys are carrying out their normal homeostatic function, very large variations of urinary potassium may occur in response to the need for conservation or elimination of potassium, with slight variations in the level of potassium in the serum. On the other hand, if the kidneys are responsible for the disturbance, the urinary excretion may be changed in an inappropriate direction, so that, far from compensating for the disturbance of serum potassium, they may be aggravating the trouble.

Most of the potassium in the body is held within the cells by forces which Professor Conway and other members of this group will discuss. It appears that the serum potassium concentration is not the only decisive factor determining the movements of potassium into and out of the cells. From the available potassium passing through the body each day, the cells withdraw the quantities necessary to carry on their functions of growth, storage, or metabolism. Any excess is released. The volume and composition of the extracellular fluids are too small and too constant to affect the external balance of potassium more than slightly or briefly. Much of our information on cellular potassium comes from a consideration of total daily balances. When a supply of potassium is presented to the cells, some estimate of their state of depletion or repletion may be made from the amount which they take up; but this is not an infallible index, since they may simply not be able to accept the added quantity at the time. It is conventional in such balances to take into account the balance of

nitrogen and any major changes in the stores of carbohydrate, so as to try to separate those exchanges of potassium which are obligatory for other metabolic functions from exchanges due to simple shifts of fluid and electrolytes.

In studying patients in a general hospital, it soon becomes evident that some degree of potassium depletion is almost universal. In part, this is due to the loss of appetite and to the poor fluid intake which is so common in seriously ill patients. Dehydration stimulates the excretion of potassium, while a poor intake of food reduces the supply. The acidosis of starvation, as well as other forms of acidosis, increases the loss of potassium in the urine. The intravenous administration of saline and glucose solutions usually leads to the excretion of still more potassium.

In acute injury to tissues, there is a characteristic release of potassium from cells into the extracellular fluid, and thence into the urine. This may in some cases be due to a simple loss of potassium from the site of injury, when large masses of muscle are involved, as in a crushing injury. In other cases, the losses of potassium are out of all proportion to the local damage, but seem to reflect a general intoxication. Such a release of cellular potassium occurs after a surgical operation, often preceding the characteristic release of nitrogen from increased catabolism of protein. It seems probable that this release of potassium from the cells is independent of renal function and of the extracellular potassium concentration, since if renal function is impaired, the serum potassium concentration may rise to very high levels at this time without any external source of potassium. When renal excretory function is normal, very large quantities of potassium may be necessary to keep the patient in balance, since much of the injected potassium is lost in the urine. A practical difficulty arises if treatment is attempted at this time, since such large quantities of potassium may be dangerous if there is even a momentary reduction of renal function. One gets the impression of fighting the natural currents of cellular release and of increased renal excretion, and quite possibly doing more harm

than good by interfering at this stage. After a few days, when the whole acute process is over, the tide turns and large quantities of potassium are retained by the cells. If adequate supplies are not available, the concentration in the extracellular fluids may fall sharply, sometimes low enough to be dangerous, and may remain subnormal until the patient starts to eat again or until enough potassium is given by vein. Here, again, the cellular activity dominates the picture, with the removal of potassium from extracellular fluids of low concentration and often with a minimum of co-operation from the kidneys, which may continue to excrete potassium because of other obligatory functions.

The magnitude of the excretion of potassium after trauma or operation is roughly proportional to the severity of the injury. It is almost certainly a part of the general, non-specific reaction to stress in which the adrenal plays such an important part. It is interesting to note that the effect of injury on potassium balance is quite comparable to the abnormality which is seen in patients with Cushing's syndrome and in other patients receiving crystalline adrenal cortical steroids or the adrenocorticotrophic hormone of the pituitary.

A sharp increase in potassium excretion follows the administration of deoxycorticosterone acetate. A similar effect occurs when ACTH is injected, while a somewhat less regular and less impressive increase in potassium excretion follows the injection of cortisone acetate. The prolonged administration of large doses of these substances frequently results in a reduction of the serum potassium level, which may be associated with clinical signs of potassium deficiency. Just as after injury, the data can be best explained by postulating both that the cells generally release potassium into the extracellular fluid and that the kidneys excrete potassium more actively. If the renal capacity for the excretion of potassium is impaired, as in the terminal stage or in some forms of the degenerative stage of nephritis, large doses of cortisone may lead to a rise in serum potassium concentration, which falls again when the hormone is withheld. On the other hand,

when renal function is normal, a large intake of potassium is necessary to maintain equilibrium in patients receiving large doses of active adrenal steroids or in patients with Cushing's syndrome. Even with a high potassium intake, the temporary balance does not necessarily mean that intracellular potassium has been restored to normal, for after withdrawal of the hormone, a further retention of potassium may be seen. If withdrawal is abrupt, surprising changes may occur. Following the removal of a tumour of the adrenal cortex in a patient with Cushing's syndrome, one might anticipate acute adrenal insufficiency with the usual increase in serum potassium. Occasionally, however, a dangerous fall in serum potassium may occur within the first few hours, reminiscent of the rapid depletion of the extracellular fluid during recovery from diabetic acidosis.

It is possible to oppose the effects of the adrenal cortical steroids to some extent by the administration of androgenic hormones which stimulate anabolic processes. Testosterone reduces the cellular losses of potassium and nitrogen which follow the stimulation of the adrenal cortex by ACTH. Some caution is necessary, however, especially in the use of combined deoxycorticosterone and testosterone treatment in adrenal insufficiency, since the stimulation both of renal excretion of potassium and of uptake of potassium by the cells may result in a serious depletion of the extracellular fluid potassium.

These interpretations are largely based on inferences, because the defects in our methods of study make direct measurements of cellular composition difficult. Analyses of tissues during potassium depletion show a reduced content of potassium, but the results are somewhat complicated by the increased volume of extracellular fluid associated with the coincident retention of sodium. The retention of sodium is usu ... ver, and it may be ... lium from the diet ... ycorticosterone acetate in arthritis, we had the opportunity to observe a

patient receiving as much as 100 mg. of DCA per day. Since this patient was taking a diet which contained virtually no sodium, there was no clinical evidence of an increased volume of the extracellular fluid, although the serum sodium concentration rose about 10 m. eq./l.; but evidences of potassium depletion appeared even though the potassium intake was maintained at a very high level.

The interaction of sodium and potassium salts remains a matter of great interest. The administration of sodium salts dilutes the extracellular potassium, increases the renal excretion of potassium, and results in a loss of cellular potassium, with some replacement by intracellular sodium. The details of this mechanism and of the apparent pharmacological antagonism of sodium and potassium are not entirely clear. Nevertheless, these actions make sodium salts useful in the treatment of potassium intoxication, but potentially dangerous when potassium stores are depleted. We have recently observed an interesting counterpart in the development of potassium intoxication in some patients suffering from severe depletion of sodium.

Several other ions affect the distribution of potassium. Darrow has demonstrated the association of potassium depletion with chloride depletion and alkalosis. The most striking clinical manifestation of this relation appears in pyloric stenosis, where the great loss of gastric secretion leads to chloride depletion and alkalosis, and then to potassium deficiency which must be dealt with before the alkalosis can be corrected.

Whether these relationships are due to conditions imposed by the kidneys or by cells generally, and what part the adrenal plays in mediation, are questions which remain to be answered. The therapeutic implications are clear, however. We must consider alkalosis and potassium deficiency together whenever either is evident. Sodium bicarbonate or lactate should not be used in the treatment of diabetic acidosis without consideration of its effects on potassium equilibrium.

In summary, it seems possible to separate to some extent the effects of the adrenal cortical steroids on the extracellular-intracellular exchange of potassium from the effects on renal excretion, by comparing their actions in patients with normal and impaired renal function. If such a separation of actions is accepted, a number of other clinical observations can be rationalized.

DISCUSSION

EICHELBERGER: I would like to ask Dr. Luetscher if in any of these patients he could possibly have taken any muscle biopsies? I think with a biopsy you could have shown what your cellular potassium was, and also what the extracellular fluids were at the time.

LUETSCHER: Analysis of muscle should give the most direct information on intracellular electrolytes. We have seldom done such analyses because of some difficulties and uncertainties. Few patients are enthusiastic about biopsy for chemical analysis The methods are tedious and therefore useful only in retrospect. Finally, there is the problem of interpretation. There seems to be much variation in the basis for expression of the analytical data. My feeling about biopsy analyses is that they must be very valuable, but that I often don't know what to make of the figures after I get them. Dr. Eichelberger has had a great deal of experience with tissue analysis. If we had her help in performing and interpreting the analyses, I am sure that we should use them often.

EICHELBERGER: As I understand it, in this type of work we have to work with patterns and methods of portrayal of these patterns. These

removal of a cortical tumour, and I should like to know if you were able to do potassium clearances before and after intervention in this case.

LUETSCHER: I don't know of any muscle analyses in familial periodic paralysis.

GROSS: You mentioned loss in plasma potassium under cortisone treatment. Contrary to this, we observed in cortisone-treated adrenalectomized dogs an increase in plasma potassium, and also signs of adrenal insufficiency. Only under simultaneous treatment with deoxycorticosterone was normal plasma potassium obtained.

LUETSCHER: Did that go on for a period of time?

GROSS Yes. After about one week of treatment with 10 mg.–25 mg. a day in dogs of about 12 kg

LUETSCHER There are many indications that deoxycorticosterone and cortisone may be different in the relative strength of their actions ... [illegible] ...bably reflect differences

... [illegible] ... Were they doing well?

GROSS Yes.

GAUNT: What happens if you just keep them on cortisone?

GROSS. They develop insufficiency within a week.

CONWAY: We found quite the opposite effect in adrenalectomized rats with cortisone There was a very substantial decrease in plasma potassium.

FOLLEY Plasma potassium would be high in untreated adrenalecto- [illegible]

... can reach nearly the same levels as in untreated animals

CONWAY. Was it with cortisone or cortisone acetate?

GROSS: Cortisone acetate, 10–25 mg per day.

[illegible]

had been given, I have no doubt that the serum potassium would have fallen

CONWAY I agree with Dr. Luetscher on that point, because in rats until we gave these large doses we did not get any effect at all. When we gave 2 mg. of cortisone acetate, which is a high dosage, then we got a fall in potassium

VERZÁR Experiments by Dr Wirz in our laboratory on adrenalectomized rats and cats showed that without treatment plasma sodium decreases and potassium increases. Daily administration of 1 or 2 mg. [illegible]

In summary, it seems possible to separate to some extent the effects of the adrenal cortical steroids on the extracellular-intracellular exchange of potassium from the effects on renal excretion, by comparing their actions in patients with normal and impaired renal function. If such a separation of actions is accepted, a number of other clinical observations can be rationalized.

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EICHELBERGER: As I understand it, in this type of work we have to work with patterns and methods of portrayal of these patterns. These

LAMOTTE: I was very interested in the potassium retention after removal of a cortical tumour, and I should like to know if you were able to do potassium clearances before and after intervention in this case.

certain psychological difficulties, not to attempt such analyses. would be most interesting.

LEWIS: You don't know if any such studies have been made, do you?

THE CONNECTION OF CARBOHYDRATE AND POTASSIUM METABOLISM, IN RELATION TO THE ADRENAL CORTEX

F VERZÁR

CHANGES in potassium and in carbohydrate metabolism are characteristic of adrenal cortical insufficiency, and corticoids restore both of them. I wish to collect in the following lines some points which prove this connection and allow certain conclusions to be drawn on the nature of the activity of the corticoids.

The following observations were made on normal animals.

(a) In muscular contraction, when glycogen is broken down, potassium is set free and can be shown in large quantities in the venous blood of the muscle (Verzár and Somogyi, 1941).

(b) After intravenous injection of glucose a hyperpotassiæmia runs parallel with the hyperglycæmia (Somogyi and Verzár, 1940).

(c) After an intravenous adrenaline injection, together with the rise in blood sugar, K also increases in the blood (Verzár and Somogyi, 1942).

All three reactions are lacking in the adrenalectomized animal. If the connection in the case of (a) could be criticized on the basis of asthenia, this is hardly possible for (b) and (c).

We have then shown (Verzár and Pulver, 1940, 1941) that when yeast produces glycogen from glucose, potassium is absorbed. When the glucose was fermented, the K became free again. In the same year, we showed this also for (horse) leucocytes, a fact which has remained, it seems, unknown and might therefore be reproduced here (Fig. 1). Lately we have shown the same also for the isolated muscle (diaphragm of rat and abdominal muscle of the mouse) (Verzár and Leupin, 1950).

and potassium in the plasma with cortisone as well as with deoxycorticosterone. Cortisone acts quickly and disappears quickly, while DCA acts slowly.

WINTER: In coronary patients the so-called side effects of cortisone are often those which are seen in potassium deficiency, and sometimes patients require additional potassium while they are under treatment with cortisone.

LOLTSCHER: Potassium deficiency occurs with prolonged use of very large dosage. The response to chronic administration of these steroids varies a great deal from patient to patient, and one cannot predict that such a change will occur; but it occurs in a certain proportion of the patients who are given moderate doses for a long period of time.

WINTER: The psychotic phenomena which have been occasionally observed under treatment with ACTH or with cortisone have been, at least in some cases, successfully combated by giving rather larger doses of potassium. Also electrocardiographic changes sometimes indicate low potassium.

ZUCKERMAN: Are these psychotic side effects of cortisone more or less universal?

WINTER: Oh no, they occur only in an occasional patient.

ZUCKERMAN: But sufficiently frequently for you to attach significance [illegible] symptoms?

[illegible]

at the same time as the potassium depletion, and that one could see changes in the electroencephalogram which were also occurring at [illegible]

psychotic manifestations which follow cortisone or ACTH are related to potassium deficiency, because we have seen them occur very rapidly in patients who were on a high intake of potassium, and without any changes in the serum potassium level. It seems that mental disturbances may be a possible manifestation of potassium deficiency, but I doubt very much that this is the regular or ordinary cause of the psychological disturbances

Verzár and Pulver (1940) had found that iodoacetic acid inhibits the K uptake of cells. This was confirmed by Rothstein and Enns (1946) and by Orskov (1948) who found that NaF and urethane did not inhibit this reaction. Many other substances which this cell metabolizes, such as acetic acid, acetaldehyde and ethylene, produce K uptake also, but only after a longer latency. Cowie, Roberts and Roberts (1949) in two papers, have treated the problem extensively with *B. coli comm.* They used radioisotope ^{42}K and confirmed all our former findings. K can be replaced by Rubidium. Their main result is that K is needed during glucose-glycogen metabolism to produce fructose-1-6-di-potassium phosphate, the reaction between glucose-6-phosphate and phosphopyruvate. They contradict Conway's view that an H-ion exchange explains the phenomenon. Muntz (1947) had supposed from his experiments that K^+ is needed to activate an enzyme to produce fructose-1-6-phosphate. But since 2 K^+ are taken up per one mol glucose, Cowie and Roberts believe that K forms the diphosphate in this reaction.

Thus the phenomenon, seen by us in leucocytes, yeast and muscle, is of general biological importance. Potassium is taken up with production of energy in cell metabolism. I myself have emphasized (1943) that the meaning of this reaction might be the production of "bound" potassium in muscle. In the myofibrils, glycogen and potassium form a complex with the proteins of the myosin group, and the production of this complex might be the point of this reaction. In other cells potassium is related mainly to the factors of the excitation process. If this is so, the disturbance of this basic process of carbohydrate metabolism, linked with K metabolism, must lead in adrenal cortical insufficiency to disturbances of muscular activity (adynamia, asthenia).

We have called attention (Verzár, 1950) to the connection of these findings with the curious fact that corticosteroids, mainly cortisone, which increase glucose and glycogen production, influence rheumatoid arthritis, a disease of local muscular origin, in such a favourable way, as is now generally

This coupling of glycogen production with potassium uptake which Verzár and Pulver (1940) found with isolated cells, was confirmed by Leibowitz and Kupermintz (1942) with *B. coli*, by Dixon (1949) in brain tissue, and by Danowski (1941) and Harris (1941) in erythrocytes.

Conway and Breen (1945) replaced K by NH_3 in yeast. It is certainly a speciality of the yeast cell that in spite of this it continues its normal carbohydrate metabolism. Conway and Malley (1946) gave an explanation of this reaction on the basis of an exchange of H^+ with K^+, and a lively discussion started about this mechanism.

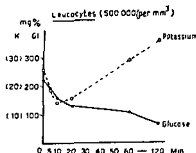


FIG 1. Uptake and release of glucose and potassium by leucocytes *in vitro*.
Between 0 and 10 min. glycogen is produced from glucose in the leucocytes (after Willstatter and Rohdewald) which is then fermented to lactic acid
(After Verzár, J., and Pulver, R., 1940.)

Muntz (1947) working with a yeast autolysate found that if neither K^+ nor NH_3^+ was present, glucose was only fermented to the hexose-monophosphate stage. Lasnitczki and Szorényi (1935) had already found an increase of fermentation with K^+ and Farmer and Jones (1942) found the same for NH_3^+. J[illegible] K^+ is necessary [illegible] and Ziegler [illegible] ntz therefore rejected the rôle of K in this reaction.

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DISCUSSION

CONWAY I can go a certain distance with Prof Verzár, and then we part company. When one considers the entrance of potassium ions into muscle, they must necessarily be balanced electrically by anions inside (or else of course the potassium must be largely bound in some unknown way, but that it is not so bound I think is obvious from the effects of osmotic pressure and many other considerations). Therefore, if there is an increase or a decrease in these non-diffusible anions, we must necessarily get an increase and a decrease in the potassium One might perhaps divide the question in two, and take on the one hand potassium permeability, and on the other hand the potassium net increase or decrease The latter is our immediate concern Now it is clear that if there are any marked metabolic changes inside the fibre, as occur when glucose is being rapidly used or stored, you get a change in the non-diffusible anions You get a change in the hexose esters,

accepted. The extraordinarily quick reaction after cortisone is a proof that the immobilization of joints was by a reflex motor-inhibition. While rheumatoid arthritis is certainly not of adrenal origin, we have called attention to muscular pain which occurs in adrenal cortical insufficiency also (Hedinger, 1950; Thorn, 1949). Free potassium in muscle is extremely painful (Baumer, 1924; Habler and Hummel, 1928; Moore and Singleton, 1934; Harpuder and Stein, 1943; Fleckenstein, 1950). We have therefore put forward the working hypothesis that a local disturbance of the enzyme reaction which fixes potassium in the muscle exists in rheumatoid arthritis. This causes pain and reflex inhibition and this can be restored to normal with adrenal corticosteroids, leading to quick changes of activity. I mention this as a sideline which shows where an understanding of the connection of these enzyme reactions with cortical hormones might lead in the future.

Summary

The fact which I wish to emphasize is that the uptake of potassium is coupled with the energy production of cells. Glycogen production leads to potassium uptake and fixation in the cell. The mechanism is still unsolved. It might be simply an ionic exchange; or a process of formation of a dipotassium-fructose-1,6-phosphate; or even a transfer of potassium to enter into complexes with special cellular proteins. This process seems to be disturbed in adrenal cortical insufficiency, and is stimulated by corticosteroids.

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to show that potassium ions can move in and out passively in relation to the accumulation or decrease of non-diffusible anions. I am in agreement so far, but I do think that although Professor Verzár may not have explicitly stated it, he seems to imply that potassium ions do have some, shall we say mysterious influence on carbohydrate metabolism.

Verzár: No!

Conway: But I am firm on that point of the exchange of potassium and hydrogen ions in yeast during short period fermentation. I do think that process is quite a different one from the accumulation of potassium ions in muscle.

Verzár: I would like to say that I never looked upon experiments on the yeast cell as anything but a model, and I certainly would not say that what is true in the yeast cell is true in muscle. That the yeast cell is totally different, Professor Conway has shown by the exchange of potassium with ammonia in the yeast cell. No warm-blooded animal cell can do that. But also with muscle and in leucocytes, we come to the result that potassium uptake goes parallel with glycogen production, while potassium liberation from the cells is associated with glycogen breakdown.

I think one should always remember that there are two parallel things going on in the cells, diffusion and metabolic processes. In the experiments on sugar absorption from the intestine, we could never get free from the simple osmotic diffusion processes, which depend every moment on the osmotic pressure inside and outside the cells. But besides these, there are the synthetic processes inside the cell which might modify osmosis.

Cole: With regard to the general proposition that is being discussed, it seems to me that if you consider this question of the relation of electrolyte balance between the cells and their environment to any metabolic changes which may occur within the cells; if you take the interrelationship between them, it seems to me that ultimately the answer must always be yes. Because if you change, to start with, the free energy of the electrolyte aspect of the system, that change, either uptake or expenditure, must come from somewhere. It obviously can't come from the electrolyte shifts, it must presumably come from some phase of organic metabolism. Secondly, it seems that if we have some active process going on in the tissues (for example, as Prof. Conway proposes, continual sodium extrusion in the cells) then that again

of some of these hormones, supposing the act...

phospho-creatine and adenosine triphosphate and you may conceivably [illegible]

Prof. Verzár gave a number of instances which he endeavoured to unify and to simplify, but to my mind it is an over-simplification to consider the rapid entrance of potassium into the yeast cells during short period fermentation as being equivalent to its entrance into muscle. He also referred to different explanations of this potassium shift and said that I had put forward a theory that it is due to interchange of potassium ions and hydrogen ions. It is not a theory; it is a fact, for the conditions studied. If one has outside the yeast 5 per cent glucose and N/10 potassium chloride with equal volumes of yeast cells and suspending fluid, then if the composition of the outside fluid be determined after 40 minutes fermentation, one may find an increase of approximately 20 m.eq. of free hydrogen ions, and a corresponding disappearance of 20 m.eq of potassium ions. I don't see how there can be any other explanation in such simple conditions where ions must balance each other, than that the hydrogen ions have exchanged for potassium. While potassium ions exchange for hydrogen ions there is an indirect and equivalent alkalinization inside the cells. N/500 azide is sufficient to cut out all this process but does not influence the overall carbohydrate metabolism, except to increase it. Further, when potassium enters the yeast cell in such a manner it is mainly balanced inside the cell by bicarbonate ions. The circumstances of rapid entry of potassium into the yeast cell are obviously very different from the phenomena in muscle. To link the two together is scarcely justified In conclusion, therefore, I am glad to say that we are in agreement concerning a general relation of carbohydrate metabolism to potassium exchanges but disagree on some related matters.

ZUCKERMAN: Professor Conway, in this dividing of the ways, do you reject some of the evidence which Professor Verzár has brought forward or do you reject part of his main proposition?

CONWAY: I reject the proposition of the unification of this problem as a single process.

ZUCKERMAN: Can you agree with any part of the proposition? If I understood Professor Verzár correctly, he was demonstrating that changes in potassium can be indirectly related to other metabolic changes. In the same way he provided us with some evidence to show that changes in cell-water occur in parallel to these other metabolic changes. Some of his evidence I understand you accept; some you reject. You reject that on the yeast Do you in consequence reject his main proposition that the shift in potassium is an indirect conse- [illegible] metabolic changes?

generally insoluble in water, we have used them in the form of a hydrosol easily obtained by addition of a sufficient quantity of Tween 80 (between 10 and 20 per cent by weight of the volume of preparation, according to cases).

We will set forth later the results obtained on groups of six rabbits. Sometimes an increase (rare), sometimes a fall of alkaline reserve has been noted; this fall, moreover, is only transitory and is followed directly by an increase. As we will show later, the rapid increase of alkaline reserve can be related to an alkalotic state and its initial fall to an acidotic state.

On account of its being hydrophilic, the rôle played by cholesterol in tissue hydration had been one of the first observed, the lipocytic ratio (cholesterol/fatty acids) controlling the quantity of water absorbed by a tissue. Since, so far as we know, its possible influence on mineral metabolism had never been demonstrated, we considered that it would be interesting to begin our researches by study of this substance.

As can be seen in Table I, our efforts were immediately rewarded, since intravenous injection of 2·5 cg. of cholesterol

Table I

INTRAVENOUS INJECTION OF 2 5 CG. OF CHOLESTEROL

	Alkaline reserve		
	Before injection	*6 to 8 hours after*	*24 hours after*
Rabbit No. 1	43 3	44 3	45 3
Rabbit No 2	30 9	36 6	34 9
Rabbit No. 3	44 4	46 2	50 0
Rabbit No 4	42 8	52 6	51 9
Rabbit No 5	32 8	32 8	32 8
Rabbit No. 6	35 0	38 5	40 4
Averages	38 2	41 8	42 5

was soon followed by an increase in the alkaline reserve, an increase which was maintained on the average over 24 hours.

These results seemed to point to an alkalinic action of the cholesterol. We had previously shown (Lecoq, 1943) that, on

STEROIDS AND ACID-BASE EQUILIBRIUM

RAOUL LECOQ

WATER metabolism depends on steroids. This is today a well established idea and abundant proof has been given of this intervention by workers in many countries. But the displacement of water usually observed in endocrine dysfunction which entails insufficient or excessive production of certain steroids, cannot be imagined without a disturbance in the metabolism of salts associated with water in its mobilization. Therefore it appears to us difficult to discuss the action of steroids on water metabolism without speaking at the same time about the jointly occurring modifications of salt metabolism.

We have already shown in a study of the metabolism of lipids (Lecoq *et al.*, 1950, 1951) that hormones leading to clinical manifestations of the same type (steatosis) produce them in very different ways, for example through an alkalotic or acidotic medium.

One could suppose that it would perhaps be the same with the hydrating action exercised by the principal steroids, and consider if salts accompanying water in its mobilization are not dependent upon acid-base disturbances. This is what we will investigate in this work. We will study the effects on the alkaline reserve of cholesterol, calciferol, dihydrofolliculine, testosterone, progesterone, deoxycorticosterone and natural 11-corticosteroids of the adrenal cortex.

The technique employed is very simple. We have injected into the marginal vein of the ear of a rabbit an aqueous preparation of the substance to be studied, and we have determined, by the van Slyke method, the alkaline reserve of the blood obtained by cardiac puncture before the injection, and 6 to 8 hours and 24 hours after the injection. Steroids being

generally insoluble in water, we have used them in the form of a hydrosol easily obtained by addition of a sufficient quantity of Tween 80 (between 10 and 20 per cent by weight of the volume of preparation, according to cases).

We will set forth later the results obtained on groups of six rabbits. Sometimes an increase (rare), sometimes a fall of alkaline reserve has been noted; this fall, moreover, is only transitory and is followed directly by an increase. As we will show later, the rapid increase of alkaline reserve can be related to an alkalotic state and its initial fall to an acidotic state.

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These results seemed to point to an alkalinic action of the cholesterol. We had previously shown (Lecoq, 1943) that, on

the contrary, calciferol (antirachitic vitamin D, acting as a regulator of phospho-calcium metabolism) seems acidotic since it prevents or cures bony lesions of classical experimental rickets developed in darkness, the character of which is undeniably alkalotic. However, we could not, by intravenous injections in moderate doses of calciferol diluted in alcohol, provoke a marked disturbance of blood alkaline reserve (Lecoq, 1946). We therefore carried out new trials, injecting in the form of hydrosol a dose of calciferol equal in weight to that of cholesterol. The modifications observed, which are recorded in Table II, were very marked, the fall of alkaline reserve still increasing after 6 to 8 hours in almost all the experiments. It must be said, however, that the metabolism of our animals was so much disturbed that in consequence more than half of them died (Lecoq, 1950).

Table II

INTRAVENOUS INJECTION OF 2 5 CG. OF CALCIFEROL

	Alkaline reserve		
	Before injection	*6 to 8 hours after*	*24 hours after*
Rabbit No 1	57 6	48 1	38 5
Rabbit No 2	48 1	40 4	43 5
Rabbit No 3	51 7	50 0	44 3
Rabbit No 4	42 8	38·5	41 4
Rabbit No 5	50 0	46 2	41 6
Rabbit No 6	42 2	40·4	38 5
Averages	48·7	43 9	41·3

It was then logical to ask oneself if cholesterol (with its probable alkalotic action) would also be different from the other steroids. Up to the present, we have only found the action of dihydrofolliculine or œstradiol (see Table III), comparable with the action of cholesterol. However, it is known that the hydrating power of this substance, belonging to the œstrane group, is one of the highest and its clinical action one of the clearest (Cachera, 1947).

Table III

INTRAVENOUS INJECTION OF 1 MG. OF DIHYDROFOLLICULINE

	Alkaline reserve		
	Before injection	6 to 8 hours after	24 hours after
Rabbit No 1	40 4	45 3	44 3
Rabbit No 2	37 0	48 1	42 4
Rabbit No. 3	50 0	50 0	51·9
Rabbit No. 4	44 3	42 4	40 2
Rabbit No. 5	30 5	34 9	32 7
Rabbit No 6	36 6	38 5	45 3
Averages	39 8	43 2	43 8

This fact being established (Lecoq, 1949), we have, with P. Chauchard and H. Mazoué (1950), verified that here it is a true alkalotic action, because the injection of this substance in the rat inhibits nervous disturbances usually following an acidogenic salt injection, like ammonium chloride. Since then, with the same authors, we have found an identical action after cholesterol injection.

On the other hand, we have studied steroids belonging to the androstane group: testosterone, and especially the pregnane group steroids progesterone, deoxycorticosterone and adrenocortical steroids (11-corticosteroids)

The hydrating power of testosterone and progesterone is markedly inferior to that of dihydrofolliculine. Since the action of these two substances on the blood alkaline reserve is inverse to the action of cholesterol and dihydrofolliculine (œstradiol), one can suppose that this is related to differences in the mechanism of action. Perhaps it could be suggested, considering our experiences with calciferol, that mineral metabolism is more important than water metabolism.

However that may be, there will be found in Table IV the blood alkaline reserve disturbances observed after intravenous injection of testosterone.

The decrease of alkaline reserve, very marked 6 to 8 hours after injection, is followed by a return to the normal, obvious after 24 hours.

Table IV

INTRAVENOUS INJECTION OF 1 MG. TESTOSTERONE

	Alkaline reserve		
	Before injection	*6 to 8 hours after*	*24 hours after*
Rabbit No 1 .	36 6	27 1	31 9
Rabbit No. 2 . .	46 2	36 6	44 3
Rabbit No. 3	39 2	30 5	36 8
Rabbit No. 4	35 4	26 6	30·2
Rabbit No. 5 . .	44·3	37 2	40·4
Rabbit No. 6 . .	48 1	32·5	36·6
Averages .	41 6	31 8	36 7

The injection of a 1 mg. dose of progesterone having provoked a still more rapid return to the normal and overshooting the initial level, we have injected higher doses of this substance. The results shown in Table V show that the equilibrium is then less disturbed and, in all cases, the alkaline reserve level found at the end of the experiment is near to the initial level.

Table V

INTRAVENOUS INJECTION OF 2·5 MG OF PROGESTERONE

	Alkaline reserve		
	Before injection	*6 to 8 hours after*	*24 hours after*
Rabbit No. 1 .	53 8	38·5	45·3
Rabbit No 2	41 4	29 6	44 3
Rabbit No. 3 . .	41 3	39 5	42 6
Rabbit No. 4 .	43 5	34 9	45 3
Rabbit No 5 . . .	39 0	36 6	36 6
Rabbit No 6 . .	40 4	37 6	42 2
Averages . . .	43 7	36 1	42 7

It will not be surprising that deoxycorticosterone and adreno-cortical extract possess an action comparable to that of the preceding substances (rapid fall of alkaline reserve); but high doses of synthetic substances show (see Table VI) a more

prolonged action (spread over 24 hours). Natural corticosteroids (in doses ten times less), cause, as is seen in Table VII, in the same period, a fall and afterwards an increase of alkaline reserve level compared with the initial level. An analogous observation had previously been made with progesterone, of which we know the explanation, durable results necessitating the use of rather high doses. But that was with pure progesterone and it is not proved that it is the same with an adreno-cortical extract in which 11-corticosteroids are accompanied by numerous other substances.

Table VI

INTRAVENOUS INJECTION OF 2 5 MG. OF DEOXYCORTICOSTERONE

	Alkaline reserve		
	Before injection	*6 to 8 hours after*	*24 hours after*
Rabbit No 1	46 2	38 5	38 5
Rabbit No 2	35 2	32 6	38 6
Rabbit No. 3	40 4	40 4	36 6
Rabbit No. 4	42 4	36 6	40 0
Rabbit No 5	34 3	32 8	30 4
Rabbit No 6	36 9	30 9	34·9
Averages	39 2	35 3	36 5

Table VII

INTRAVENOUS INJECTION OF ADRENO-CORTICAL EXTRACT CONTAINING 0·25 MG. OF TOTAL 11-CORTICOSTEROIDS

	Alkaline reserve		
	Before injection	*6 to 8 hours after*	*24 hours after*
Rabbit No 1	38 5	29 0	46·2
Rabbit No 2	38 8	36 6	36·6
Rabbit No. 3	40 6	30 5	50·0
Rabbit No 4	38 8	38 8	30·9
Rabbit No 5	44 6	30 4	42 4
Rabbit No 6	38·5	31·9	48·1
Averages	39 9	34·5	42·3

I have only performed such measurements on animals receiving ricketogenic diets, and the changes of the tissue pH were closely connected with the modifications of the alkaline reserve of the blood.

COLE. I am wondering if it would be possible to get even more specific [illegible] In measuring reserves

increases the alkali reserve, and testosterone depresses it. But can we correlate that with anything that we know of the action of testosterone on renal excretion, or migration of ions from the tissue fluids into the cells?

HELLER I should like to ask Dr. Lecoq whether he relates the changes in the renal excretion of electrolytes under the influence of steroids to the tissue changes which he has found?

LECOQ: There is a dissociation between the tissue changes and the renal changes and therefore I have not included the renal effects in my considerations.

CONWAY: The matters Dr. Lecoq has raised are obviously of great interest, and their further elucidation cannot fail to have value. As Dr. Cole pointed out, it is a question of changes of bicarbonate in blood and very many things might change that bicarbonate. It would appear that one outstanding possibility is the coming out of acidic products into the blood, and if so, an analysis of the blood for fatty acids and such metabolic products would be desirable.

THE INTERRELATIONSHIP BETWEEN THE ADRENAL CORTEX, POSTERIOR PITUITARY AND ANTERIOR PITUITARY IN WATER METABOLISM*

ROBERT GAUNT

Several months ago when I was asked for a topic on which I might lead a discussion, I proposed a rather broad one. Now that I am faced with the job of saying something about it in 25 minutes I am going to find it difficult to deal with it adequately.

Since my colleague, Dr. Birnie, is here to pick up the loose ends, however, I shall present briefly a somewhat hypothetical, but I hope not entirely fanciful scheme of suggested relationships between the adrenal cortex, the posterior pituitary and the anterior pituitary as they relate to the regulation of water metabolism. The work which I shall discuss from our [illegible] 1949) have covered the literature on this subject and no extensive bibliography will be included here.

For brevity I shall deal only with those phases of the subject which directly or indirectly relate to the kidney and thus affect water excretion. In doing so it should be kept in mind, however, that we are overlooking extra-renal actions of these hormones of great importance.

The Adrenal Cortex

The hormones of the adrenal cortex may, depending upon physiological circumstances, cause either a water retention or

*This work was aided by grants from the National Heart Institute, U.S. Public Health Service and Ciba Pharmaceutical Products, Inc.

an acceleration of water excretion (Gaunt *et al.*, 1949). Similarly they may either retard or enhance sodium excretion. It would simplify matters if it could be said that their effect on water excretion was dependent on and proportional to their effect on sodium excretion. But while that sometimes seems to be true it is not always the case (e.g. Green *et al*, 1930). No complete correlation of these facts is possible at the present time and none will be attempted here. When water retention is seen, however, as an action of cortical hormones it seems to be a consequence of sodium retention, whereas the diuretic action of cortical hormones, on the other hand, may involve other mechanisms, and not be dependent on sodium diuresis.

We will concentrate here on the nature of the diuretic influences of adrenal cortical hormones. There are two well-established facts, the possible causes of which provide the basis for this discussion. (*a*) Adrenalectomized animals or Addisonian patients cannot excrete a water load rapidly and the former show a high susceptibility to water intoxication. The latter is partly due to the deficient diuresis and partly to other causes. (*b*) Cortical hormones given to water-loaded normal animals increase the rate of diuresis and protect against water intoxication.

Renal Factors

Removal of the adrenals generally leads to a decreased glomerular filtration rate. When existent this factor presumably contributes to the sluggish diuretic response to water. Decreased filtration is not an essential component of the deficiency in handling water, however, as seen by the fact that replacement therapies which maintain the filtration rate do not necessarily permit the occurrence of normal water diuresis (Lotspeich, 1949; Roemmelt *et al.*, 1949). The more important factor seems to be an enhanced tubular reabsorption of water. This has been seen by many workers and in our experience has been an invariable accompaniment of adrenal insufficiency.

The augmented rate of urine flow that follows the administration of cortical hormones to normal animals is not associated with any essential change in the glomerular filtration rate. Various workers have found this rate to be either slightly increased, decreased or unaffected. This means that the diuretic action of the corticoids is achieved by a depression of the tubular reabsorption of water—a finding which might be anticipated from those mentioned above in adrenal insufficiency.

From the large amount of data in the literature on these points, I have selected as representative excerpts from the tables of Boss *et al.* (1950)—Table I.

Table I

G.F.R. AND URINE FLOW IN WATER-LOADED RATS

(Mean values on groups of 8 to 20 animals)

Treatment	*G F R*	*Urine Flow*
	(ml /100 gm /min)	
None—normal	1 00	0 06
Adrenalectomized Animals		
Adx —7 days	70*	0 005*
Adx —7 days. 0 5 mg DCA/day	84	0 02*
Adx.—5 days. 5 0 mg DCA/day	84*	0 06
Intact Animals		
DCA—5 mg	0 90	0 075*
A C E —4 ml	0 99	0 076*

*Significantly different from normal

Factors that may Cause an Increase in the Tubular Reabsorption of Water in Adrenal Insufficiency

In adrenal insufficiency the homeostatic mechanisms which normally provide a sensitive regulation of the rate of water excretion obviously break down. The renal tubules avidly

reabsorb water even if to do so results in water intoxication. There are various possible causes of this phenomenon.

(*a*) Several laboratories (Martin *et al.*, 1939; Birnie *et al.*, 1950; Lloyd and Lobotsky, 1950) have now found that there is an increased amount of some antidiuretic substance in the blood and urine after adrenalectomy, a substance which has actions similar to the antidiuretic hormone of the posterior pituitary.

(*b*) Shorr and co-workers (1950) have recently reported that the antidiuretic substance, ferritin, increases in the blood after adrenalectomy. Its possible relation to the agents mentioned above have not been investigated.

(*c*) There is evidence of a hypersensitivity to posterior pituitary hormones after adrenalectomy (Birnie *et al.*, 1950), due presumably to the absence of antagonizing cortical hormones, which may be a factor of great importance. Dr. Lockett's paper in this symposium provides conclusive additional evidence of a hypersensitivity to posterior lobe principles after adrenal ablation.

In any case, it is probable that this behaviour of the renal tubules in the absence of cortical hormones is induced by a humoral agent.

Factors Causing Accumulation of Antidiuretic Agents in Body Fluids after Adrenalectomy

In trying to explain the accumulation of antidiuretic agents in body fluids after adrenalectomy, one thinks first of possible hypersecretion of the posterior pituitary antidiuretic hormone. There is no direct physiological evidence on whether this does or does not occur; cytological evidence indicates that it does not (Gersh and Grollman, 1939). On theoretical grounds hypersecretion might not be expected, particularly after water-loading, because of the reduction of plasma sodium salts induced by water administration in adrenalectomized animals. If the osmotic pressure of plasma is similarly reduced, ADH secretion should be suppressed rather than stimulated. The possibility exists, nevertheless, that some

stimulant (such as ferritin?) appears in the blood after adrenalectomy and causes the release of ADH.

On a more positive note, one demonstrated fact is that the livers of adrenalectomized rats are unable to inactivate vasopressin as effectively *in vitro* as are those of normal animals. Thus, there could be an accumulation of ADH because of decreased destruction rather than over-production. This places focus on the possible important rôle of the liver in this whole problem, a subject that will be discussed subsequently in this conference by Dr. Birnie.

Is the Induction of Water Diuresis Normally Associated with Hypersecretion of Cortical Hormone?

Satisfactory evidence has been provided by Verney (1947) and others that excess water is excreted rapidly due to suppression of ADH secretion. The work discussed above, however, shows that excess water cannot be excreted rapidly in the absence of adrenal cortical hormones. That raises the question, is water diuresis normally associated with *increased* adrenal cortical activity as well as *decreased* ADH secretion? Dr. Nagareda (1951) in our laboratory has investigated this question by imposing various fluid loads on rats and measuring changes in adrenal ascorbic acid as an index of adrenal stimulation. She found in brief that a water load approximating to 5 ml. per 100 gm. body weight (actually 3 ml. per 100 sq. cm. of body surface), when given by stomach tube, produced significant adrenal ascorbic acid depletion beyond that due to any of the manipulative procedures. Larger doses of water had greater effects. A smaller dose (1·5 ml. per 100 sq. cm. of body surface) produced no detectable adrenal stimulation by this method. It is an open question whether or not a more sensitive method would have revealed any effect with the light water loads. The work of Lloyd and co-workers (1950) in man and of the Danfords (1950) in rabbits indicates that water diuresis is associated with a high rate of excretion of corticoid metabolites. This is generally interpreted to mean a high rate of corticoid secretion. From all this it is safe

to conclude that at least a high rate of water diuresis is associated with both a suppression of ADH release and a stimulation of the adrenal cortex.

Normal saline, unlike distilled water, did not evoke adrenal ascorbic acid depletion even when given in huge dosage. This shows that the adrenal was not being stimulated by fluid loads as such. It might be that after the administration of distilled water the adrenal was responding, in a converse manner, to the same stimulus which acts on the posterior pituitary, namely, changes in the osmotic pressure of plasma. Definite evidence on that point is being sought.

The Anterior Pituitary

An extensive literature on the endocrine regulation of water metabolism assigns some diuretic rôle to the anterior lobe of the pituitary (Gaunt *et al.*, 1949; Pickford and Watt, 1950). A strong argument can be made out that that influence is *mediated in part through the adrenal cortex* by ACTH. On the other hand, considerable work indicates that certain gross abnormalities in renal function of hypophysectomized animals cannot be due to loss of the ACTH—adrenal cortical system (White *et al.*, 1949; de Bodo *et al.*, 1950).

One clear fact is that the diuretic response to water is equally or more retarded after hypophysectomy than after adrenalectomy (Chen and Geiling, 1943; Joseph *et al.*, 1944, Pickford and Watt, 1950). This deficiency, unlike other renal deficiencies, can be at least partially repaired by adrenal cortical extract (Joseph *et al.*, 1944). It is difficult if not impossible, however, to restore a normal ability to handle a water load in hypophysectomized animals by administering adrenal cortical hormones. Drs. Boss and Osborn (1950) in our laboratory are attempting to analyse further the nature of the possible deficiency in the hypophysectomized rat and the replacement value of adrenal cortical hormones. Table II gives some of their representative data. Control animals in their experiments received normal saline because the adrenal cortical extract used was made up in isotonic sodium chloride.

speak, see behind the sometimes hackneyed words and the jargon which editorial activity imposes on people, their personality coming through. We shall be able to do that now. We shall read Dr. Samuel's paper and hear his slow voice and very acute ideas coming through. We shall think of Dr. Dorfman surrounded by masses of steroid formulæ, darting rapidly here and there, and we shall think of him replacing those parallel lines by arrows as data accumulate.

I do not know to what extent the infra-red will penetrate the editorial blue pencil, but when we read in his papers of removal of the side chain we shall know what Dr. Dobriner is saying to himself!

Finally, it is my extremely pleasant duty to record our gratitude to the Ciba Foundation and personally to Dr. Wolstenholme for the excellent arrangements he has made for our comfort and instruction. He has kept us in good spirits throughout these discussions.

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show an antidiuretic response, suggesting that even though the concentration of unstable ADS in jugular blood may be quite high, the arterial blood when reaching the kidney contains too little antidiuretic activity to be demonstrated with our method of assay.

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DISCUSSION

ZUCKERMAN. Dr. Heller, would you explain what happens to the [illegible]

and arterial plasma, and in both of these types of blood we could not find any antidiuretic activity. That doesn't say that there isn't any; it only means that our methods of assay cannot detect very small amounts. It seems to me quite logical that there is in venous outflow from the head a relatively high concentration of posterior pituitary antidiuretic hormone, but that then it is diluted by the rest of the blood in the body. Inactivation of part of the secreted hormone and renal excretion have also to be considered.

GAUNT. We are delighted to see these results. Various people have found confusing things about these circulating antidiuretic materials, and I believe this work gives more order and understanding than we have had before.

O'CONNOR: I would like to ask Professor Heller or Dr Gaunt about the amount of the substance that is circulating. From the little I know of the assay method using the rat, the amounts of antidiuretic hormone appearing in the venous blood must be quite enormous relative to the amounts that have ever been involved in the experiments of Professor Verney or myself on dogs in Cambridge The amounts that we found were all, in fact, extremely small and would be quite unassayable, as far as I can see, by the rat method of assay

HELLER. Any amounts that we find in jugular blood, Dr. O'Connor, are also fractions of milli-units There are various reasons why one would expect the concentration of antidiuretic hormone to be high in the jugular blood of our rats. First, these rats were not particularly hydrated Secondly, the blood was being collected from animals under ether anæsthesia, and we don't know what effect that has. [illegible]

them with our methods of assay. Though I don't know what sort of blood you refer to in dogs (and you have seen from this how important it is to distinguish between jugular blood and vena cava blood, arterial blood, and so on), one would expect that the blood coming from the pituitary would contain more than the peripheral blood, enough perhaps to be analysable by the rat method.

LEWIS: I would like to ask Dr. O'Connor whether he has read the [illegible]

to get enough hormone to handle chemically. When I was asking Dr. Heller my question I was thinking not of any direct assay of hormone in blood, but the amount deduced from comparisons of the antidiuretic

if there is any method of assay by which we can hope to detect them *in the jugular blood. So that the demonstration of antidiuretic sub*stance in blood, so far as I can see, can only be achieved under conditions which have, in fact, nothing to do with the ordinary function of the pituitary gland.

GAUNT Your estimates in the dog agree, if I remember correctly, with those arrived at by Shannon.

O'CONNOR: Yes.

HORMONAL INFLUENCES ON THE WATER METABOLISM OF NEW-BORN AND YOUNG ANIMALS

H. HELLER

The study of the water and mineral metabolism of new-born infants and animals has attracted more and more attention during the past ten years. Many aspects of the problems involved have been successfully investigated and numerous differences between new-born and adults have been discovered. However, comparatively little work has been done on the new-born to elucidate the rôle of those endocrine glands which like the posterior pituitary and the adrenal cortex are known to have a profound influence on the water metabolism of the adult. Investigations concerned with these questions have been proceeding for some time in my laboratory and some information has been gathered. I should like to make a brief survey of the results so far obtained.

When working with new-born rats, one is impressed with their apparent inability to concentrate their urine or its individual constituents to the same degree as the adult. Apparently quite highly concentrated urines are sometimes obtained but the *mean* concentrations or osmotic pressures are undoubtedly much below those of adult rats. This contrast becomes even clearer when adults and new-born are made to undergo a comparable degree of dehydration (Heller, 1949). Why do the new-born rats excrete less concentrated urines than do adults? This may be partly due to a low rate of glomerular filtration leading to increased reabsorption of urinary crystalloids (Pitts and Duggan, 1950; Bradley, Mudge and Blake, 1950; Blake, Wegria, Ward and Frank, 1950) and may perhaps also be due to a lack of tubular secretion of potassium (Heller, 1951), but important as these considerations may be, they

hardly supply the full answer. When new-born rats are deprived of fluid for 24 hours one finds (Heller, 1949) that in contrast to adults, the losses of body water lead to considerable hæmoconcentration and to decreases of tissue water, or in other words to a significant contraction of the extracellular fluid phase. Now one mechanism by which the adult animal, made to undergo a comparable degree of fluid loss, counteracts such a state, is the elaboration of a concentrated urine. It is generally agreed that it does so by reabsorbing more water under the influence of the posterior pituitary antidiuretic hormone. Does this mechanism also apply to the new-born animal or to what degree does it apply? Conditions for the action of the antidiuretic hormone on the tubular urine of the new-born are, if anything, more favourable than in the adult, as less water has to be reabsorbed per unit time at lower than at higher rates of tubular urine flow to achieve the same concentration. The question therefore arises to what degree the posterior pituitary renal mechanism operates in the new-born. Figs. 1 and 2 show the results of injections of vasopressin on the urine volume and the inulin U/P ratio of new-born rats to which 4·5 per cent of their body weight of water had been given by stomach tube. The demonstration of an antidiuretic effect in new-born rats is *a priori* not easy, mainly because rats of that age do not empty their bladder spontaneously. An experimental procedure was therefore devised which, though differing from the usual technique of measuring antidiuretic effects, permits one to decide whether the new-born rat responds in a similar manner as the adult. A comparatively large dose of vasopressin—10 mU/100 g. rat—was injected into adult controls and it was found that with this dose the inhibition of diuresis lasted for approximately 145 minutes. The same relative doses of vasopressin and of water were given to the new-borns. The animals were killed 145 minutes later, the urine was removed by bladder puncture and then weighed on an analytical balance. It was known from other experiments (Heller, 1947*a*) that 145 minutes are sufficient to permit the absorption

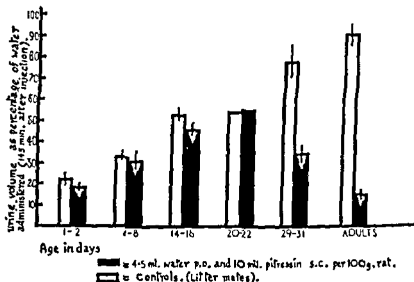


Fig. 1. The effects of an injection of vasopressin on the urine volume of adult and infant rats. Each column represents the mean of results obtained in at least 10 animals. (Heller, unpublished experiments.)

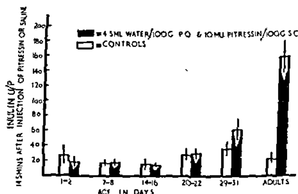


Fig. 2. The effect of an injection of vasopressin on the inulin urine/plasma ratio of adult and infant rats (Heller, unpublished experiments.)

of the water administered to new-born rats. It will be seen from Fig. 1 that the response of the new-born is different from that of the adult controls. Similar experiments on rats aged 1, 2, 3 and 4 weeks showed that significant decreases in urine volume were only obtainable in rats older than three weeks. Estimations of inulin U/P ratios showed much the same. It seems quite possible that a response to the antidiuretic hormone will be demonstrable in somewhat younger rats if more refined methods are used, but there is little doubt from the results presented that the full antidiuretic effect is only established some considerable time after birth. There are additional reasons why one would expect this to be so. Determinations of antidiuretic hormone in the neurohypophysis (Fig. 3) showed that the gland of new-born rats contains very much less of this active principle than that of adults (Heller, 1947*b*). There is therefore the suspicion that in the new-born rat there is not only an incomplete renal response to the antidiuretic hormone but also that there is not enough hormone available to act.

I am not sure whether this latter consideration is applicable to new-born infants. Per mg. gland the posterior pituitary of new-born infants contains only about one-fifth of the hormone content of adults (Fig. 3) but the total amounts present are quite respectable. There is, however, no doubt (Fig. 4) that new-born infants even when deprived of fluid do not achieve the same urinary concentrations as adults. There may then be sufficient antidiuretic hormone in the gland but are adequate amounts released? And if so, does the kidney respond as in the adult? We have, as yet, no information on the rate of release in the new-born but the response to injected posterior pituitary extract has been tested. It will be seen from Fig. 5 that infants react to large doses of injected posterior pituitary extract—an increase of osmolar concentration by over 100 per cent can be seen in one of the experiments—but the rise was neither as marked nor was the duration of the effect as lengthy as in the adult controls which had received an equivalent dose of the antidiuretic hormone. Similar experiments on

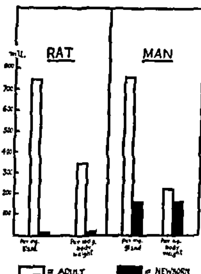


FIG 3. The quantities of antidiuretic hormone present in the pituitary in the adult and new-born of rat and man (Based on the results of Heller, 1947*b*, and Heller and Zaimis, 1949.)

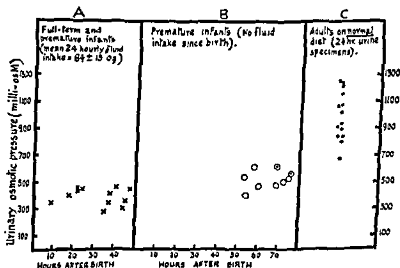


FIG. 4. Urinary osmotic pressures in infants and adults (A and C based on the paper by Heller, 1944, B on that of Smith *et al*, 1949)

new-born infants have been briefly reported by Barnett, Hare, McNamara and Hare (1948) and they agree with my findings to the extent that *some* effect of large intravenous doses of vasopressin on the inulin U/P ratio could be demonstrated in premature infants. Unfortunately, however, Barnett and his co-workers do not seem to have done any control experiments on adults. It is, therefore not possible to use their results for

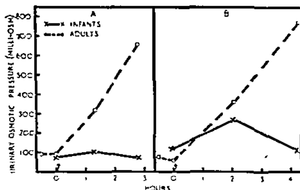


FIG. 5 The effect of equivalent doses of Posterior Pituitary Extract (B D H) on the urinary osmotic pressure of infants

a quantitative assessment of the differences between the responses of the neonatal and the adult kidney.

While a fair amount of work has thus been done, I feel that I should emphasize here that our work on the response of new-borns to the antidiuretic hormone is by no means completed. Some controls are still missing, such as for instance an enquiry into the rate of inactivation of the antidiuretic principle in the body of new-born animals. There also remains another feature which I find puzzling, namely the relationship between the development of the diuretic response to water and the antidiuretic response to the hormone.

Because of its clinical implications, it would seem to be well worth while to establish with clarity the function of the neurohypophysio-renal mechanisms in the new-born. Dehydration is undoubtedly a serious problem in early infancy; it remains a contributary cause of the continued high fatality in cases of infantile diarrhœa and vomiting. It appears quite likely that the new-born have some defence against the renal loss of water, a decrease of GFR for instance would lead to increased retention of body water though only at the price of retaining metabolic end products and electrolytes. Clearly, however, as long as the infant does not concentrate its urine to the same degree as adults there is the risk that water will be lost in the urine at times when utmost economy in the handling of this body constituent would be indicated.

It should perhaps be added that the results of this enquiry into the function of the posterior pituitary-renal mechanism at birth may have some bearing on a pathological condition in older children and adults which has been termed "nephrogenic" or "pituitrin-resistant" diabetes insipidus. Recently published cases (Forssman, 1945; Williams and Henry, 1947; Dancis, Birminghams and Leslie, 1948) show this in some instances to be a congenital and hereditary defect. It seems quite possible that here we are dealing with a condition in which the development of the kidney is in some respects arrested at the level of that of a new-born infant, or judging from the report of complete absence of response to large doses of vasopressin, perhaps even at an antenatal level. The actual site or nature of renal abnormality remains for the present an open question. It seems preferable, however, to restrict the term diabetes insipidus to cases where insufficient secretion of the antidiuretic hormone has been demonstrated and to regard the condition just described as a separate pathological entity.

The question of the diuretic response of the new-born mammal to administered water has already been mentioned. It has been shown by McCance and Wilkinson (1947) and by

myself (Heller, 1947a) that the urine flow is little increased when a dose of water is given to new-born rats. In fact, it seems from McCance's and our finding that in this species the adult rate of diuresis is only reached during the third or fourth week of postnatal life. Water diuresis in the new-born of mammalian species which are more mature at birth than the rat is somewhat better developed but it is noticeable that even new-born guinea-pigs, which are more developed at birth than the rat, the dog or the infant, and whose kidney function has been shown to be in many respects similar to that of the adult, appear to be unable to excrete administered water at the adult rate (Dicker and Heller, 1951). Water diuresis in the new-born infant has as yet not been fully investigated but there are indications that it resembles that of new-born animals.

It seems likely that there are quite a number of factors concerned in this inability of new-born and young animals to handle administered water in the same manner as adults but only one factor will here be discussed. Gaunt and his co-workers have shown that the response to administered water is deficient in adrenalectomized animals. They have also demonstrated that the injection of adrenal cortical extracts markedly enhances water diuresis. It seemed of interest therefore to find out whether a similar rise in the renal output of administered water could be induced by giving adrenal cortical extracts to new-born and young rats. Doctor Birnie and I have started to investigate this matter and several series of experiments on rats aged 1, 2, 3 and 7 weeks have been completed. Fig. 6 shows that a dose of 2 ml./100 g. of Upjohn cortical extract does not accelerate or increase the output of urine in rats aged 1 or 2 weeks. A significant effect was, however, obtained in animals aged 3 weeks and over. Further experiments with deoxycorticosterone and with cortisone are under way. Ultimately it is hoped to use ACTH but we shall only be able to do so when purer preparations will be available. Control experiments on adult rats with the purest preparation at our disposal—for which we have to thank Dr. Max Reiss of

Bristol—showed antidiuretic activity of an intensity which excludes its use in experiments concerned with renal water and electrolyte excretion.

It should be added that some preliminary findings on the effect of adrenal cortical extracts on the new-born have recently been published by Osborne and LoCascio (1950). The authors claim that adrenal cortical extracts enhance urine excretion in rats aged only 24 to 48 hours. Our experiments do not support this claim, in fact it seems to us very doubtful

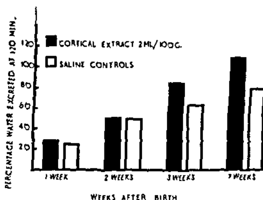


FIG 6. Effects of administration of Adrenal Cortical Extract (Upjohn) on the urine volume of infant rats. The blocks represent the mean of results obtained in at least ten animals.

whether results of any reliability can be obtained with a method using body weight loss as an index of urine excretion. Such factors as the high extrarenal water loss (Heller, 1947*a*) and the presence of residual urine have clearly to be considered. We do not wish to imply by saying this that we doubt the possibility of hormonal effects on *some* bodily functions in rats younger than three weeks. Indeed Table I shows experimental evidence to that effect. We regard it as likely, however, and this assumption is supported by the morphological investigations of Yoffey and Baxter (1948), that the kidney of very young rats is too immature to respond to either the adrenal or the posterior pituitary hormones.

Table I

MATURATION OF RATS IN TERMS OF RESPONSES TO VARIOUS STIMULI

	A	B	C D E	F G H	I J K	L
0		7	14	21		28

Days after Birth

A = Adrenal enlargement by chronic ACTH administration (Moon, 1940)
B = Adrenal ascorbic acid depletion in response to adrenaline (Jailer, 1949)
C = Ovaries first stimulated by sex hormones (Corey, 1928)
D = First protective action against cold stress by cortical hormone (Holtkamp *et al.*, 1949)
E = Adrenal ascorbic acid depletion in response to cold stress (Jailer, 1950)
F = Attainment of ability to regulate body temperature (Hill, 1947)
G = Adult-like response of the testis to gonadotrophins (Smith and Engle, 1927)
H = Diuretic response to cortical extract (Birnie and Heller, unpublished)
I = Antidiuretic response to vasopressin (Heller, unpublished)
J = Loss of resistance to cold (Adolph, 1948)
K = Loss of resistance to anoxia (Antoschkina, 1939)
L = Adult like type of diuresis in response to administered water (Heller, unpublished)

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THE INFLUENCE OF SODIUM CHLORIDE ON WATER DIURESIS AND ITS RELATION TO THE ADRENAL

H. W. HAYS

Introduction

It is well recognized that adrenalectomized animals and the Addisonian fail to conserve sodium chloride and are unable to excrete ingested water at a normal rate. Although it has been generally agreed that failure to conserve sodium is due to lack of hormonal regulation, there is little agreement as to the nature of this defect.

There are conflicting opinions regarding the rôle of sodium chloride in cardiac failure and hypertension, but as shown by Merrill (1949), Kempner (1949), Grollman *et al.* (1945), and Schroeder (1949), there is little doubt that diets low in sodium may be beneficial, on the other hand, Schroeder has observed renal failure on such diets and this is referred to as a low salt syndrome.

Shortly after the introduction of mercurial diuretics, it became evident that there were certain potential dangers to the use of these drugs, particularly salt depletion and dehydration.

In 1944, Thorn, Koepf and Clinton described a condition of salt-losing nephritis which simulated adrenal cortical insufficiency, many of the symptoms could be attributed to the low sodium levels.

Most clinical work has attempted to point out the value of diets low in sodium for treatment of congestive failure and in hypertension, but at the same time Soloff and Zatuchni (1949) have pointed out the danger of affecting renal function. Little experimental data is available on this mechanism. Before

discussing this problem I would like to mention several notable observations which perhaps focused our attention more directly on electrolytes and water diuresis: (1) As shown by Swingle *et al.* (1937), Hays and Mathieson (1945), and Gaunt *et al.* (1949), water alone does not restore diuresis in adrenalectomized animals. (2) Deoxycorticosterone acetate causes a retention of sodium in normal as well as adrenalectomized animals, and as reported by Swingle *et al.* (1941) and Hays *et al.* (1945), may occur in the latter with a rapid increase in plasma volume. (3) It has been demonstrated by many (Swingle and Remington Review, 1944) that sodium chloride will maintain the life of adrenalectomized animals for a considerable period of time, and increasing the amount of sodium chloride in the diet of Addisonians decreases the amount of deoxycorticosterone acetate required. (4) The clinical syndrome of heat cramps simulates the condition seen in adrenalectomized animals given forced water.

Methods

(A) *Salt Depletion Studies*

(1) *Dogs.* A five per cent glucose solution was administered intraperitoneally under pressure by the method of Darrow and Yannet (1935) and in an amount equal to 100 cc./kg. of body weight. After five hours the fluid was removed by paracentesis under pentothal anæsthesia. The dogs were then maintained on a low salt diet.*

(2) *Rats.* A five per cent glucose solution was given intraperitoneally with a syringe and in a volume equivalent to ten per cent of the body weight. After three hours the fluid was removed by paracentesis and the animals placed on low salt diet. Rats weighing 200–300 gm. were used in all experiments

(B) Hydration

(1) *Dogs.* Water was administered by mouth in the amount of 25 cc./kg. of body weight at hourly intervals. Urine volumes were determined prior to each intubation of water. The percentage excreted was calculated on the basis of the total water administered.

(2) *Rats.* Water or sodium chloride was intubated at four hourly intervals in an amount equal to six per cent of the body weight. Urine volumes were determined at hourly intervals for a period of seven hours and the percentage excreted calculated on the basis of the total water administered.

(C) Chemical Determination

Blood was withdrawn from dogs before and after hydration; in rats, only at the end of seven hours. Serum sodium concentrations were determined by the Perkin-Elmer flame photometer; serum chloride by Eisenman's modification of the Volhard-Sendroy method.

Results

(A) Effect of Water in Normal and Adrenalectomized Rats

Normal rats excreted 75–80 per cent of the water administered, while rats adrenalectomized for sixteen hours excreted only 46 per cent. This latter value is somewhat higher than previously reported, but may be explained in part by differences in strains and methods of handling. The normal animals remained free of symptoms, while adrenalectomized controls showed marked symptoms of water intoxication and many died from such treatment (Table I).

(B) Effect of Varying Salt Concentration

It will be observed in Tables I and II, and Fig. 1 that when increasing concentrations of salt solutions are given to normal animals there is a progressive decrease in diuresis. This difference in response to the various salt solutions is evident at the fifth and seventh hours.

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* ... papers have appeared in the

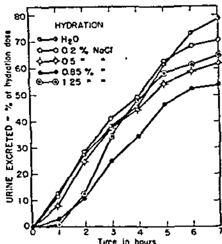


FIG. 1. Effect on diuresis in normal animals of hydration with water and various concentrations of salt solutions

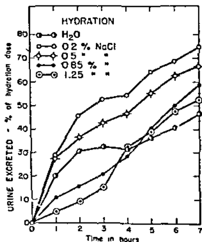


FIG. 2. Effect on diuresis in animals adrenalectomized for 16 hours of hydration with water and various concentrations of salt solutions.